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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

In year 2 of our studies of the inhibitory interaction between matriptase and HAI1, and between matriptase and BBI, we have built a 3-D structure of the protease domain of matriptase, based on the homology modeling using the X-ray structure of human thrombin as template. This modeled matriptase structure was used in a structure-based screening of inhibitors. Screening from the NCI small compounds database, we have developed bis-benzamidines as potent matriptase inhibitors. We have also found that, in addition to HAI1, a recently discovered natural trypsin inhibitor, SFTI, from sunflower seed, inhibits matriptase. We have found that in non-transformed mammary epithelial cells, the activated matriptase can be stimulated by lipid phosphates; the activated matriptase is then quickly bound to HAI1, and shed into media. Thus, engagement of membrane-bound matriptase with HAI1 leads to its extracellular shedding, not its cellular internalization. In addition, we have identified hepatocyte growth factor and pro-uPA as likely physiological protein substrates of matriptase.

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INTRODUCTION

Recently, we have identified in human breast cancer a new epithelial-derived, type 2 integral membrane, serine protease, matriptase, and its cognate inhibitor, the hepatocyte growth factor activator inhibitor 1 (HAI1) [4-6]. In the current research plan, we proposed to study the inhibitory interactions between matriptase and HAI1, and between matriptase and the Bowman-Birk Inhibitor (BBI). In the first year, we completed and reported parts of Aims 1 and 2. We reported the purification of matriptase and HAI1 from human milk, the enzymatic specificity of matriptase, and a controllable system for matriptase activation in epithelial cell culture. We also reported on an application of a molecular modeling approach for inhibitor screening. In our second year, we have made further progress in this study; results are presented in this report.

STATEMENT OF WORK

Aim 1: We will first establish in detail the mechanism and kinetics in solution of inhibition of the 80-kDa protease by the BBI, compared to the Kunitz domain inhibitor (KSPI, HAI1) we have previously identified. **COMPLETED**.

Aim 2: We will next examine whether the BBI, compared to KSPI (HAI-1) is capable of binding the membrane-bound 80-kDa protease and promoting its cellular internalization. As described in the current report, we have found that engagement of membrane-bound matriptase with HAI1 leads to its extracellular shedding, rather than its cellular internalization, as initially hypothesized. Therefore, **we have shifted our focus to study the shedding of matriptase and matriptase/HAI-1 complexes**, since the answer to the question posed in Aim 2 is negative.

Aim 3: We will test the ability of the BBI to influence proliferation and differentiation through the c-myc system.

BODY

In the period of July, 2000-June, 2001, we have completed the studies in Aim 1, and have made interesting findings in the studies for Aim 2.

Aim 1: We will first establish in detail the mechanism and kinetics in solution of inhibition of the 80-kDa protease by the BBI compared to the Kunitz domain inhibitor (KSPI) we have previously identified.

We completed most of this aim and reported our findings in previous year. As described earlier, we utilized molecular modeling, instead of phage display, to optimize inhibitor discovery, based on BBI and HAI1 inhibitory domains. In the period of July 2000-June 2001, we have continued to make significant progress in these studies. We published these studies in two manuscripts.

One manuscript **"Structure-based approach for the discovery of bis-benzamidines as novel inhibitors of matriptase"** has been published **in the Journal of Medicinal Chemistry** (reprint included in APPENDICES). As we showed in this paper, the 3D structure of the protease domain of matriptase was first built with homology modeling using the X-ray structure of human thrombin as template. The modeled structure of the protease domain of matriptase (figure 1B), by analogy to thrombin, has a catalytic triad positioned on the surface, marked by Ser805, His656, and Asp711, corresponding to Ser195, His57 and Asp102 in thrombin. Consistent with the observation that matriptase prefers substrates with an Arg or Lys as P1 residue [3;5;11], a negatively charged residue, Asp799, is located at the bottom of the S1 binding site (figure 1B). This modeled structure was then used to screen small compounds in the NCI database [9]. Since the S1 site is considered to be the primary substrate binding site in serine proteases, it is likely to be a good target site for inhibitor design [1;2;10]. Also, since the S1 site of matriptase is negatively charged, the potential inhibitor candidates that target this site should be positively charged in water under physiologic conditions. In addition, other two putative binding sites, the anionic site and the hydrophobic S1' site, were included in the docking site used for 3D-database searching with the program DOCK [8]. Using these criteria, a total of 69 candidate compounds were selected from the best scoring 2,000 compounds. Table 1 in the paper shows that among these 69 compounds, 15 of them, at 75 μM concentration, inhibited more than 95% of the

protease activity. These 15 compounds were analyzed further for their K_i values. One group of compounds that we have identified from this screening is bis-benzamidines. As shown in table 2 in the paper, the 7 bis-benzamidines analogs available from the NCI database have Ki values ranged from 191 nM to greater than 10 μM against matriptase. Compounds 2 and 7 are the most potent inhibitors for matripatase. Both compounds are also selective to matriptase compared with uPA and thrombin; compound 7 is more selective than compound 2 is (table 2 in the paper).

In addition to small compound inhibitors, molecular modeling also revealed a naturally occurring small peptide inhibitor, termed sunflower trypsin inhibitor (SFTI) [7], to be a good inhibitor of matriptase. SFTI was initially purified as an actual product from sunflower seeds. It is a 14-aa backbone-cyclized peptide that is further stabilized by an intramolecular cystine disulfide bond. SFTI has been shown to be a potent and selective inhibitor of trypsin [7]. By using synthetic SFTI peptides, we found that it inhibited matriptase with Ki of 0.92 nM, stronger inhibition than that observed with BBI. As we stated in previous report, the estimated Ki for HAI1, purified from the human milk, is 1nM. Thus, SFTI appears to inhibit matriptase as efficiently as HAI1, the cognate inhibitor of matriptase. In addition to its potency, SFTI is also selective. It has little inhibition to thrombin, and no inhibition to uPA. We have submitted these studies in a manuscript **"Synthesis and evaluation of the sunflower derived trypsin inhibitor as a potent inhibitor of the type II transmembrane serin protease, matriptase"**, and it is in press in the journal **Bioorganic and Medicinal chemistry Letters** (see APPENDICES).

Aim 2: We will next examine whether the BBI compared to KSPI (HAI-1) are capable of binding the membrane-bound 80-kDa protease and promoting its cellular internalization. In previous year, we reported a controllable model for serum-dependent activation of matriptase on the living mammary epithelial cells. In the past year, we have identified that sphingosine 1 phosphate (S-1-P) is the major blood-borne factor to induce matriptase activation in non-transformed mammary epithelial cells (Fig. 1). In

this controllable model, we discovered that activated matriptase is quickly bound to HAI-1, and the matriptase-HAI-1 complex is shed into media. These observations suggest that ectodomain shedding is the major way to remove the activated matriptase from cell surfaces. Although these discoveries could not fully exclude the possibility of internalization of matriptase/HAI-1 complex, removal of matriptase by ectodomain shedding appears to account for the fate of the most majority of the inhibitory bound protease, this process could prevent unwanted proteolysis on cell surfaces. This hypothesis is supported by our early observation in which matriptase and matriptase/HAI-1 complexes were identified and purified from the conditioned media of breast cancer cells and mammary epithelial cells and from human milk. Therefore, in this aim, we have emphasized study the shedding of matriptase and matriptase/HAI-1 complexes, instead of the originally proposed internalization of matriptase. We have further found that matriptase is constitutively present in the activated form in human breast cancer cells. Apparently, breast cancer cells have developed an autonomous way to activate matriptase and have escaped the control by S-1-P. Up to five-fold increased level of activated matriptase in complex with HAI-1 was shed into media by MDA MB 486 breast cancer cells, compared to non-transformed A1N4 mammary epithelial cells grown in the absence of S-1-P (Fig. 2).

Furthermore, we have identified HGF and pro-uPA as the substrates of matriptase. We showed in our paper **"Activation of hepatocyte growth factor and urokinase type plasminogen activator by matriptase, an epithelial membrane serine protease"**, published in **J. Biol. Chem.**, that HGF is cleaved into two chains after incubation with active matriptase (see number 1 in APPENDICES). This cleaved HGF can stimulate scattering of Madin-Darby canine kidney epithelial cell line and tyrosine phosphorylation of the receptor c-Met in A549 human lung carcinoma cell. Also shown in this publication is the fact that matripatase can proteolytically convert pro-uPA into the protease active, two-chain form of uPA that is able to cleave its specific peptide substrate. Matriptase, however, does not cleave plasminogen, despite the high amino

acid sequence homology between HGF and plasminogen. Matriptase does not directly cleave other extracellular matrix proteins such as collagens, fibronectin, and laminin (data not shown). However, it is likely that matriptase serves to indirectly cleave these proteins through its activation of pro-uPA and possibly other proteases.

KEY RESEARCH ACCOMPLISHMENTS:

In the second year, we have:

- Built a modeled structure of protease domain of matriptase, and found it to be suitable in structure-based screening of inhibitors.
- Identified a group of bis-benzamidines as potent small compound inhibitors of matriptase.
- Shown that iodo and bromo substituents on the benzamidine phenyl rings of these inhibitor compounds can improve their selectivity.
- Developed two bis-benzamidine derivatives that can be used in studies in cell culture systems and possibly in an *in vivo* system.
- Shown that SFTI inhibits matriptase activity as well as HAI1.
- Shown that SFTI has little to no inhibition of thrombin and uPA.
- Shown that activation of matriptase in normal mammary epithelial cels can be stimulated by S-1-P, resulting in extracellular shedding, rather their intracellular internalization.
- Found that matriptase in breast cancer cells is present primarily in its activated forms. These cells do not response to S-1-P stimulation, and cell constitutively release the matritpase complexes into their extracellular media.
- Identified HGF and pro-uPA as biological substrate of matriptase.

REPORTABLE OUTCOMES:

ABSTRACTS:

Oberst, M., Johnson, M., Amders. J., Dickson, R. B., and Lin, C.-Y., 2000, Full length cloning of the serine protease matriptase and characterization of its expression in breast cancer primary tumors, metastasis, and cell lines. AACR, 91st Annual Meeting. San Francisco, CA

Lee, S.-L., Dickson, R. B., and Lin, C.-Y., 2000, Biological function of matriptase, a novel trypsin-like protease. FACS/The Salk Institute for biological studies. 16th Annual Meeting on Oncogenes and tumor suppressor. La Jolla, CA

Enyedy, I., Lee, S.-L., Lin, C.-Y., Dickson, R. B., and Wang, S., 2000, Structure based design of inhibitors for matriptase. Proceeding of the Annual meeting of the American Chemical Society. Washington, DC

Roller, P. P., Long, Y.-Q., Li, P., Lee, S.-L., Lin, C.-Y., Enyedy, I., Wang, S., and Dickson, R. B. 2001, Bicyclic peptide inhibitors of an epithelial cell-derived transmembrane protease, matriptase. Proceeding of the 17th American Peptide Symposium. San Diego, CA

PUBLICATIONS:

1. Lee, S.-L., Dickson, R.B., and Lin, C.-Y., 2000, Activation of hepatocyte growth factor and urokinase type plasminogen activator by matriptase, an epithelial membrane serime protease. J. Biol. Chem. 275, 36720-36725.

2. Enyedy, I.J., Lee, S.-L., Kuo, A.H., Dickson, R.B., Lin, C.-Y., and Wang, S., 2001, Structure-based approach for the discovery of bis-benzamidines as novel inhibitors of matriptase. J. Medicinal Chem. 44, 1349-1355.

3. Long, Y.-Q., Lee, S.-L., Lin, C.-Y., Enyedy, I., Wang, S., Dickson, R.B., and Roller P.P., 2001, Synthesis and evaluation of the sunflower derived trypsin inhibitor as a potent inhibitor of the type II transmembrane serine protease, matriptase. Bioorganic & Medical Chemistry Letters. In press.

4. Benaud, C., Dickson, R.B., and Lin, C.-Y., 2001 Regulation of the axtivity of matriptase on epithelial cell surfaces by a blood-derived factor. Eur. J. Biochem. 268, 1439-1477.

5. Roller, P.P., Long, Y.-Q., Li, P.; Lee, S.-L., Lin, C.-Y., Enyedy, I.;Wang, S., Dickson, R.B., 2001, Bicyclic peptide inhibitors of an epithelial cell-derived transmembrane protease, matriptase. In 'Peptides: The Wave of the Future' (Proceedings of the 2nd International / 17th American Peptide Symposium), R.A. Houghten and M. Lebl (Eds.). In press.

CONCLUSION:

In our second year, continuing our molecular modeling approach, we have built a 3-D structure model of the protease domain of matriptase based on the homology modeling with the X-ray structure of human thrombin as template. Using the modeled matriptase structure, we have developed bis-benzamidines as potent and selective matriptase inhibitors. Using the same molecular modeling approach, we have also found that the BBI-related, natural trypsin inhibitor, SFTI, from sunflower, as well as its endogenous inhibitor HAI1 inhibits matriptase, with Ki values in the sub-nanomolar

range. These results emphasized that molecular modeling is capable of being used in searching for an inhibitor of a protein without an established x-ray structure. From our studies, this approach resulted in identification of potent inhibitors that have the potential to be used in *in vivo* assay, and possibly, in clinical application. We showed that matriptase can be activated in normal breast cells by S1P; but matriptase is constitutively activated in breast cancer cells, independent of exogenous S1P. We have found that matriptase is the activator of HGF and pro-uPA, but not of plasminogen; it also does not appear to directly cleave extracellular matrix proteins. These results suggest that matriptase may be an important upstream activator in cancer cell pericellular proteolysis and migration. These studies may result in a new therapeutic approach to breast cancer.

Fig. 1: LPA and S1P induce the activation of matriptase.
Serum starved 184A1N4 cells were treated for 1 hour with the indicated concentrations of LPA (**A**), SIP, sphingosine (1□g/ml), ceramide (1□g/ml) or ceramide-1-phosphate (1□g/ml) (**B**). **C,** Serum starved 184A1N4 cells were treated for the indicated amount of time with 10 ng/ml S1P. Equal amounts of whole cell lysates were analyzed by Western with mAb M69 (activated matriptase) and mAb M32 (total matriptase).

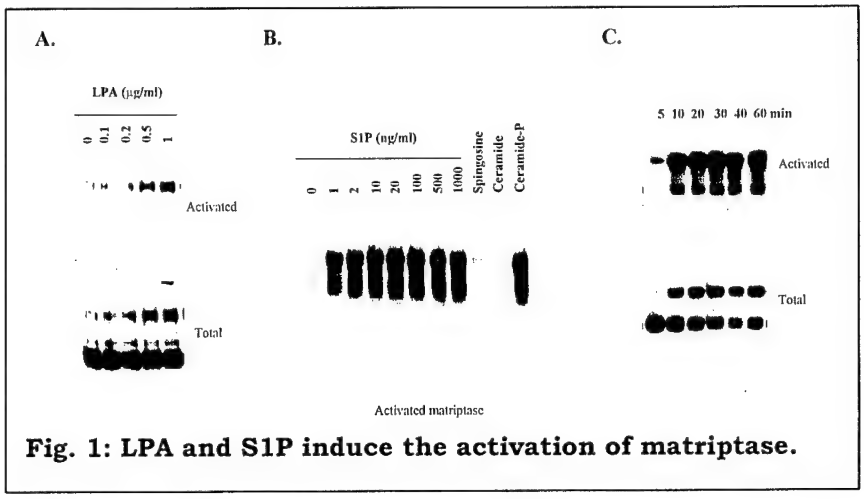

Fig. 1: LPA and S1P induce the activation of matriptase.

Fig. 2. Increased ectodomain shedding of matriptase in breast cancer cells. A and B, Equal numbers of non-tumorgenic human mammary epithelial cells (184 A1N4) and human breast cancer cells (MDA MB 468, MCF-7, and SkBr 3) were serum-starved, and then maintained in the presence or absence of 50 nM S1P for 24 hr. The media were collected and concentrated. Equal amounts of media were examined by immunoblot using anti-total matriptase mAb M32 (A) and anti-two-chain matriptase mAb M69 (B). C, To compare the amounts of matriptase released into the media in the absence of S1P between immortalized non-tumorgenic mammary epithelial cells and the breast cancer cell lines, equal volumes of the conditioned media for each cell lines were analyzed by western blotting for the presence of activated (mAb M69) and total matriptse (mAb M32). Western blot results were analyzed by densitometry analysis, and the values obtained for each cell line were normalized to the total amount cellular proteins present in the tissue culture plate at the time the conditioned media was collected. (a.u., densitometry arbitrary units)

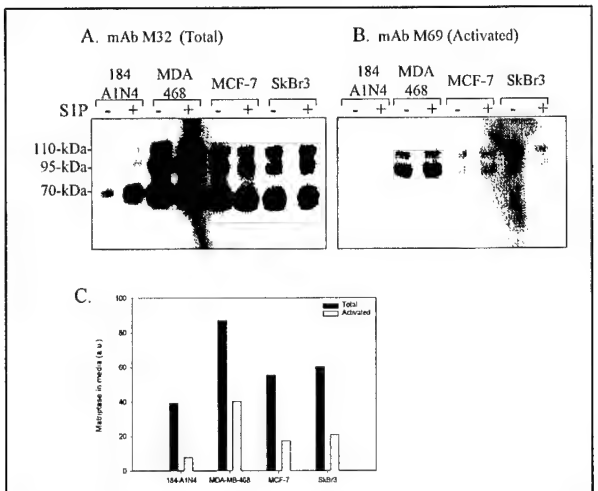

Fig. 2. Increased ectodomain shedding of matriptase in breast cancer cells.

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APPENDICES

1. Lee, S.-L., Dickson, R.B., and Lin, C.-Y., 2000, Activation of hepatocyte growth factor and urokinase type plasminogen activator by matriptase, an epithelial membrane serime protease. J. Biol. Chem. 275, 36720-36725.

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Activation of Hepatocyte Growth Factor and Urokinase/Plasminogen Activator by Matriptase, an Epithelial Membrane Serine Protease*

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Matriptase is an epithelial-derived, integral membrane serine protease. The enzyme was initially isolated from human breast cancer cells and has been implicated in breast cancer invasion and metastasis. In the current study, using active matriptase isolated from human milk, we demonstrate that matriptase is able to cleave various synthetic substrates with arginine or lysine as their P1 sites and prefers small side chain amino acids, such as Ala and Gly, at P2 sites. For the most reactive substrates, *N-tert*-butoxycarbonyl (*N-t*-Boc)-γ-benzyl-Glu-Ala-Arg-7-amino-4-methylcoumarin (AMC) and *N-t*-Boc-Gln-Ala-Arg-AMC, the K_m values were determined to be 3.81 and 4.89 μM, respectively. We further demonstrated that matriptase can convert hepatocyte growth factor/scattering factor to its active form, which can induce scatter of Madin-Darby canine kidney epithelial cells and can activate c-Met tyrosine phosphorylation in A549 human lung carcinoma cells. In addition, we noted that matriptase can activate urokinase plasminogen activator but has no affect on plasminogen. These results suggest that matriptase could act as an epithelial, upstream membrane activator to recruit and activate stromal-derived downstream effectors important for extracellular matrix degradation and epithelial migration, two major events of tissue remodeling, cancer invasion, and metastasis.

Tissue remodeling is observed both in physiological and pathologic processes. These include organ development, morphogenesis, wound healing, cancer invasion, and metastasis. Degradation of extracellular matrix (ECM)[1] and cellular migration are two prominent steps in tissue remodeling. Considering that the majority of the ECM-degrading proteases, such as the plasmin/urokinase type plasminogen activator system (1), and the major motility factor, hepatocyte growth factor (HGF)/scattering factor (SF) (2) are mainly produced by the stromal components *in vivo*, tissue remodeling is likely to be an event that depends entirely upon stromal-epithelial collaboration (3). A search for epithelial-derived proteases, which may interact both with stromal-derived ECM-degrading protease systems and with motility factors, could provide a missing link in our understanding of tissue remodeling and cancer invasion and metastasis.

To investigate the epithelial role in tissue remodeling and in many aspects of tumor behavior, including growth and metastasis, we have carried out our studies on an epithelial-derived, integral membrane, trypsin-like, serine protease (matriptase) and its cognate, Kunitz-type serine protease inhibitor (hepatocyte growth factor activator inhibitor-1, HAI-1) (4–7). Matriptase is a type 2, integral membrane, trypsin-like serine protease with two putative regulatory modules: two tandem repeats of a CUB (C1r/s, Uegf, and Bone morphogenetic protein-1) domain and four tandem repeats of a low density lipoprotein (LDL) receptor domain (also see updated sequence in the GenBank™/EBI Data Bank with accession number AF118224). Matriptase was independently cloned by others, and termed membrane-type serine protease 1 (MT-SP1) (8). The mouse homologue of matriptase was also cloned and termed epithin (9). The cognate inhibitor of matriptase is a type 1 integral membrane protein, containing two Kunitz domains, separated by an LDL receptor domain (7). The inhibitor was independently characterized by others as an inhibitor (HAI-1) of hepatocyte growth factor activator, an enzyme identified in serum (10).

Considering that matriptase exhibits trypsin-like activity and presents on the surfaces of epithelial cells, and that activation of the uPA system and HGF/SF requires cleavage at Arg or Lys, we hypothesize that matriptase could act as an upstream, epithelial membrane activator of the downstream, stromal-derived effectors of tissue remodeling. In the current study, we set out to investigate the potential collaboration between epithelial and stromal cells by examining if matriptase is able to activate HGF/SF and the protease components of the uPA system. Using the 70-kDa active matriptase, purified from human milk, we are able to demonstrate that matriptase can activate pro-uPA and pro-HGF, but not plasminogen. These results reveal that a novel mechanism involving both an upstream epithelial membrane activator as well as downstream stromal effectors may play an important role in tissue remodeling.

MATERIALS AND METHODS

Antibodies—Polyclonal antibodies to hepatocyte growth factor (HGF) α-chain (C-20) and β-chain (N-19) were purchased from Santa Cruz

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[1] The abbreviations used are: ECM, extracellular matrix; HGF, hepatocyte growth factor; SF, scattering factor; HAI-1, hepatocyte growth factor activator inhibitor-1; LDL, low density lipoprotein; MT-SP1, membrane-type serine protease 1; uPA, urokinase plasminogen activator; pro-uPA, single-chain form of human uPA; MDCK, Madin-Darby canine kidney; PAGE, polyacrylamide gel electrophoresis; FCS, fetal calf serum; PBS, phosphate-buffered saline; AMC, 7-amino-4-methylcoumarin; *N-t*-Boc, *N-tert*-butoxycarbonyl.

Biotechnologies (Calne, Wilshire, UK). Monoclonal anti-human Met antibodies (clones DL-21 and DL-24) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The recombinant anti-phosphotyrosine antibody (RC20: HRPO) was from Transduction Laboratory (Lexington, KY).

Cell Lines and Protein Substrates—Human lung carcinoma cell line A549 was from the ATCC. Madin-Darby canine kidney (MDCK II) epithelial cell lines and the single-chain form HGF protein were the generous gifts from Dr. George Vande Woude (Van Andel Research Institute, Grand Rapids, MI). The single-chain form of human urokinase plasminogen activator (pro-uPA) was purchased from American Diagnostics Inc. (Greenwich, CT). Plasminogen, plasmin, and fluorescent substrate peptides *N-tert*-butoxycarbonyl (*N-t*-Boc)-Gln-Ala-Arg-7-Amido-4-methylcoumarin (AMC), *N-t*-Boc-Gln-Gly-Arg-AMC, *N-t*-Boc-Leu-Gly-Arg-AMC, *N-t*-Boc-γ-benzyl (Bz)-Glu-Gly-Arg-AMC, *N-t*-Boc-γ-Bz-Glu-Ala-Arg-AMC, *N*-succinyl (Suc)-Ala-Phe-Lys-AMC, *N*-Suc-Leu-Leu-Val-Tyr-AMC, Suc-Ala-Ala-Pro-Phe-AMC, and Suc-Ala-Ala-Ala-AMC were from Sigma Chemical Co. (St. Louis, MO).

Purification of Active Matriptase—The 70-kDa active matriptase and its endogenous inhibitor HAI-1 were purified from human milk by immunoaffinity chromatography and maintained in their uncomplexed status in glycine buffer, pH 2.4, as described previously (7). Matriptase and HAI-1 were further separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was stained using a zinc stain kit (Bio-Rad, Hercules, CA). The 70-kDa active matriptase was sliced out and then eluted from the gel using Electro-Eluter (Bio-Rad) under non-denatured conditions (Tris-glysine buffer, pH 8.3). Alternatively, the active matriptase was purified by a high pressure liquid chromatography C18 column (Vydac VHPB5). The mobile phase was A = 0.1% trifluoroacetic acid in water; B = 95% acetonitrile in water containing 0.1% trifluoroacetic acid. The flow rate was 1 ml/min. The gradient was set from 0 to 60% B over 8 min and at 60% B for next 7 min. The 70-kDa active matriptase was composed of two major and one minor cleaved products of the membrane-bound matriptase. The cleavage sites of the two major bands were identified to be Lys^{189}-Ser^{190} and Lys^{204}-Thr^{205} (the numbering of amino acid residues are based on the updated cDNA sequence of matriptase). The 70-kDa, active matriptase contains two CUB domains, four LDL receptor domains, and the serine protease domain but lacks the transmembrane domain.

Determination of Matriptase Activity—The enzyme activity of matriptase was measured at room temperature in a reaction buffer containing 100 mM Tris-HCl (pH 8.5) and 100 μg/ml bovine serum albumin, using a fluorescent peptide as substrate. In brief, 10 μl of enzyme solution and 10 μl of peptide substrate were added to a cuvette containing 180 μl of the reaction buffer. The mixture was mixed well, placed back into a fluorescent spectrophotometer (Hitachi F4500), and the release of fluorescence resulting from hydrolysis of the peptide substrate was recorded with excitation at 360 nm and emission at 480 nm.

Determination of Kinetic Parameter—Substrate concentration *versus* initial reaction velocity were analyzed by the Michaelis-Menten equation and plotted using SigmaPlot software. Double reciprocal (Lineweaver-Burk) plots thus derived were used to determine V_{max} and K_m values.

Cleavage of Protein Substrates—Single chain HGF protein, plasminogen, or pro-uPA was incubated with various amounts of matriptase in 100 mM Tris-HCl (pH 8.5) overnight at room temperature. Incubation was stopped by boiling the mixture in SDS sample buffer. The cleaved products were then separated on SDS-PAGE and analyzed by Western blot hybridization or by silver stain.

Scattering Assay—The MDCK II cell line was maintained at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). The scatter assay was carried out in the 96-well culture plate (11). To each well was added 150 μl of Dulbecco's modified Eagle's medium supplemented with 5% FCS and leupeptin at 100 μg/ml; HGF or matriptase-cleaved HGF was added into one well and serial 2-fold dilutions were made with sequential 150-μl aliquots of medium transferred from well to well. About 3000 MDCK II cells in 150 μl of medium were added to each well, and the plate was placed in a 37 °C incubator for 20–24 h. Media were removed, and cells were fixed and stained for 15 min with 5% crystal violet in 50% methanol. Cell scattering (spreading and dispersion of epithelial colonies) was examined under light microscopy.

c-Met Phosphorylation Detection—A549 cells were grown confluent in RPMI medium supplemented with 10% FCS. After 3-hour serum starvation, cells were incubated 5 min at 37 °C with 450 ng/ml HGF or matriptase-cleaved HGF in RPMI medium supplemented with 5% FCS.

TABLE I

Kinetic parameters of matriptase for various peptide substrates

10 μl of matripase solution and 10 μl of peptide substrate were added to a cuvette containing 180 μl of reaction buffer, and the release of fluorescence resulting from hydrolysis of the peptide was recorded in a fluorescence spectrophotometer at room temperature with excitation at 360 nm and emission at 480 nm. K_m and V_{max} were determined by double-reciprocal plot derived from the Michaelis-Menten equation.

Peptide substrate	P4-P3-P2-P1-AMC	K_m	V_{max}
		μM	nM AMC/min
1	Gln-Ala-Arg[a]	4.89	654
2	Glu-Ala-Arg[a]	3.81	76.3
3	Leu-Gly-Arg[a]	13.6	309
4	Gln-Gly-Arg[a]	33.5	528
5	Glu-Gly-Arg	47.5	170
6	Ala-Phe-Lys	69.9	524
7	Leu-Leu-Val-Tyr[a]	-[b]	-[b]
8	Ala-Ala-Pro-Phe[a]	-[b]	-[b]
9	Ala-Ala-Ala[a]	-[b]	-[b]

[a] Gln-Ala-Arg is a standard substrate for trypsin, Glu-Ala-Arg is a substrate for factor XIa, Leu-Gly-Arg is a substrate for uPA, Gln-Gly-Arg is a substrate for XIIa, Leu-Leu-Val-Tyr and Ala-Ala-Pro-Phe are substrates for chymotrypsin, and Ala-Ala-Ala is the substrate for elastase.

[b] No cleavage activity was detected with these substrates at a concentration of 200 μM.

As appropriate, leupeptin was included in the medium at 100 μg/ml. Media were removed, and cells were rinsed with 1× phosphate-buffered saline (PBS) and collected by centrifugation following trypsinization. After washing one more time with 1× PBS, cell pellets were frozen in dry ice. The frozen cell pellets were either stored at −80 °C for later extraction or immediately extracted as described below. Cells were thawed on ice, extracted by suspension in buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1% Triton X-100. Extracts were clarified by centrifugation for 15 min at 12,000 × *g* in a microcentrifuge, and the protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as standard. About 2 mg of protein of extracts was immunoprecipitated using anti-c-Met antibody and Pansorbin (Calbiochem, La Jolla, CA). The protein-antibody-Pansorbin immunocomplex was collected by centrifugation, washed twice with extraction buffer then with 1× PBS, and then dissociated by boiling in SDS-sample buffer. Pansorbin was removed by centrifugation, the supernatant fractions were subjected to 8% SDS-PAGE, and the proteins were detected by Western immunoblot using anti-phosphotyrosin antibody. The same immunoblots were then stripped in 100 mM 2-mercaptoethonal, 2% SDS, and 62.5 mM Tris-HCl (pH 7.6) for 30 min at 50 °C and reprobed with anti-c-Met antibody.

RESULTS

Matriptase Selectively Cleaves Peptide after an Arg or Lys Residue—In our previous study, the trypsin-like activity of matriptase was suggested by the observations that 1) an Asp residue positioned at the bottom of a substrate binding pocket of the serine protease domain of matriptase and 2) matriptase is able to cleave various synthetic substrates containing Arg or Lys as P1 sites (6). In the current study, to investigate in detail the substrate specificity of matriptase, we measured the K_m and V_{max} of matriptase for a variety of protease substrate peptides. Table I shows that the most reactive peptide substrates for matriptase are *N-t*-Boc-Gln-Ala-Arg-AMC, with a K_m of 4.89 μM, and N-t-Boc-Bz-Glu-Ala-Arg-AMC, with a K_m of 3.81 μM. *N-t*-Boc-Gln-Ala-Arg-AMC and N-t-Boc-Bz-Glu-Ala-Arg-AMC were reported to be good substrates for bovine trypsin and human factor XIa, respectively (12). No released fluorescence was detected from the substrates for chymotrypsin or elastase (Table I, peptide substrates 7, 8, and 9). Matriptase

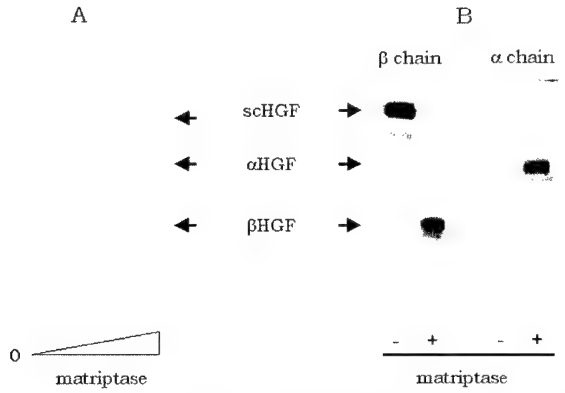

FIG. 1. **Matriptase converts single-chain HGF into smaller fragments, which can be recognized by anti-α-chain HGF and β-chain HGF antibodies.** *A*, protein staining. Shown are the silver-stained protein patterns of HGF incubated overnight without (*0*) or with increasing amount of matriptase. *B*, Western immunoblot. HGF incubated overnight without (−) or with (+) matriptase was immunoblotted with anti-βHGF (*β chain*) or anti-αHGF (*α chain*).

appears to prefer to bind to peptides containing small side-chain amino acids, such as Ala and Gly, at P2 site (Table I, peptide substrates 1–5). Peptides containing P2 Ala are better substrates for matriptase than peptides containing P2 Gly (compare peptides 1 and 2 with peptides 3–5). The binding affinity of matriptase to the former is about 30-fold higher than that to the latter. Interestingly, a change from Gln to Glu at the P3 site significant reduces the V_{max} (compare peptide 1 with 2) without causing a significant change to the K_m.

Considering its trypsin-like activity and its presentation on the surfaces of epithelial cells and cancer cells, matriptase could serve as a cell surface activator for other secreted proteases and growth factors. Particularly relevant substrates could include those factors that are produced by stromal cells, that function on epithelial cells, and that require proteolytic cleavage at Arg or Lys for their activation. We, therefore, set out to examine whether matriptase can activate three such substrates, HGF/SF, plasminogen, and pro-uPA, in addition to characterizing the synthetic substrates for matriptase.

Matriptase Is Able to Activate HGF/SF—HGF/SF is secreted as an inactive, single chain precursor by stromal cells, and it is activated by proteolytic conversion to the two-chain form factor by cleavage at Arg^{495} (13) in the extracellular environment. This cleavage is required for HGF/SF to activate its cell surface receptor c-Met. To determine if HGF/SF can be cleaved by matriptase, inactive HGF/SF, purified from the condition medium of fibroblast cells cultured in the absence of serum (a generous gift from Dr. Vande Woude, Van Andel Research Institute) was utilized in the following experiments. Fig. 1*A* shows that this HGF/SF preparation is primarily composed of the single-chain form protein, with an apparent size of about 97 kDa on SDS-PAGE (*left lane*). Because it was reported previously that there was a limited contamination of cleaved form HGF in preparations of latent form HGF (14, 15), we wished to establish whether there was contamination in our preparation of latent HGF. As expected, we also observed minor species of 64- and 33-kDa apparent molecular mass, corresponding to the expected sizes of activation cleaved α- and β-chains of HGF/SF, respectively. After incubation with the active form matriptase, the amount of 64- and 33-kDa molecules increased at the expense of a 97-kDa molecule. The lowest amount of matriptase allowing observable HGF cleavage was at a molar ratio of approximately 500:1 for HGF:matriptase. Western immunoblot analysis using monoclonal antibodies to α- and β-chain HGF showed that the 64-kDa species contains αHGF and the 33-kDa species contains βHGF (Fig. 1*B*). The contaminating 64- and 33-kDa species in the latent HGF sample in the absence of incubation with matriptase were also detected with the respective antibodies (Fig. 1*B*). The anti-αHGF antibody apparently does not recognize the single-chain form HGF/SF (Fig. 1*B*). Thus, matriptase can convert the single chain HGF/SF into fragments containing α- and β-HGF. We scanned the silver-stained protein gel and the x-ray film of this Western immunoblot by densitometry and estimated the relative amounts of the full-length, α-, and β-HGF (data not shown) using ImageQuant software (Molecular Dynamics). On average, we found that areas under the α- and β-chains were 25% and 30%, respectively, of the total stained HGF. The α- and β-chains in the original HGF preparation were 25–30% of their respective values in the matriptase-cleaved sample, as determined by both silver-staining and Western immunoblotting. Therefore, we estimated that approximately 25% of the HGF in our preparation of latent HGF is in the cleaved (activated) form.

To further investigate if the cleavage of HGF/SF by matriptase corresponds to a process of activation, we performed a well-characterized assay specific to HGF activity, the MDCK cell scatter assays (2, 11). The single-chain HGF/SF was first incubated with matriptase to allow its complete cleavage; the cleaved products were than diluted in the medium and incubated with MDCK II cells for 20–24 h at 37 °C. To eliminate the possibility of activation of HGF/SF by other activators in the serum, leupeptin was included in every scattering assay (16). It was previously observed that leupeptin can also inhibit matriptase enzyme activity (4). Fig. 2 shows the images of plates of MDCK II cells after incubation with various dilutions of untreated (HGF) or matriptase-treated (HGF/MTP) HGF/SF. In the control sample (CRL), cells aggregated into tight clusters after 20–24 h culture. In the presence of 1:4096 dilution of HGF/MTP, cell islands were more scattered, and there was a high proportion of single cells. In contrast, at the same dilution of HGF, cells were not significantly different from the control cells. The scatter morphology was more profound with progressively lower dilutions of HGF/MTP. At 1:512 dilution of HGF/MTP, cells completely separated from each other and many of them exhibited elongated, fibroblast-like shapes. On the other hand, scattering morphology induced by the untreated HGF appeared at much lower dilution (Fig. 2, HGF, 1:512). For the cells incubated with untreated HGF, we consistently observed the first sign of scatter at 1:1024 dilution, compared with 1:4096 dilution for the matriptase-cleaved HGF. This difference in the scattering activity comparing matriptase-cleaved and uncleaved HGF is very close to the difference observed in the protein level demonstrated in Fig. 1. Thus the baseline scattering activity in samples treated with uncleaved HGF apparently was derived from the contaminating active HGF in the HGF/SF preparation (Fig. 1). Cells grown in the presence of matriptase alone retained the same morphology as those in the control (data not shown). Cells incubated with untreated HGF/SF in the absence of leupeptin were examined as a positive control for HGF activation by serum; the scattering activity of HGF/SF in these samples was as expected (data not shown). These observations showed that the scattering induced by HGF/MTP is indeed enhanced by the matriptase activation of HGF.

HGF functions through binding to its cell surface receptor, c-Met. Upon binding to HGF, c-Met is activated following phosphorylation at tyrosine residues. In Fig. 3, c-Met activation was examined in the A549 human lung carcinoma cell line. Like the experiments for Fig. 2, leupeptin was also included in these treatments. Tyrosine-phosphorylated c-Met was observed in cells incubated with matriptase-cleaved HGF (HGF/MTP) and with untreated HGF (HGF), but not in cells incubated in the

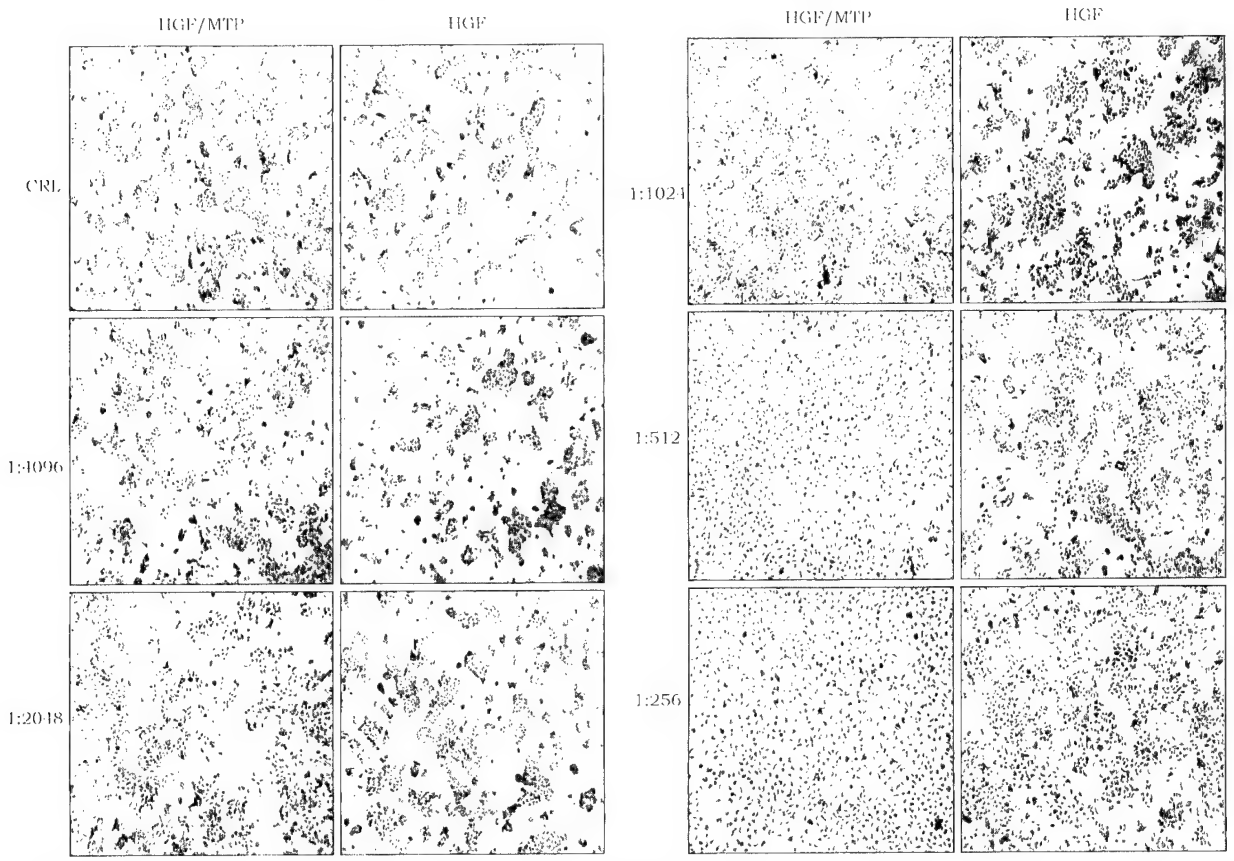

FIG. 2. **Matriptase-cleaved HGF stimulates scattering on MDCK cells.** Plates show wells in the 96-well plates with about 3000 MDCK cells after 20 h in the absence (*CRL*) or presence of matriptase-cleaved HGF (*HGF/MTP*) or untreated HGF (*HGF*) at the dilutions shown. Leupeptin was included in every culture.

absence of HGF (No HGF). The tyrosine-phosphorylated c-Met in the cells treated with uncleaved HGF is about one-fifth of that in the cells treated with matriptase-cleaved HGF. Again, consistent with a limited degree of contamination of cleaved HGF in our latent HGF preparation. Phosphotyrosine detected in c-Met of cells incubated with untreated HGF appears to be caused by the residual active HGF contamination in the preparation. Leupeptin did not affect the total c-Met expression, the c-Met phosphorylation, nor the total pattern of tyrosine phosphorylation (data not shown).

Plasminogen shares high homology with HGF, and its activation also requires a cleavage at Arg. Therefore, it seemed likely that plasminogen would be a substrate of matriptase as well. However, matriptase failed to cleave plasminogen. As shown in Fig. 4, plasminogen remained as a 94-kDa single-chain form, even in the presence of matriptase at a concentration 8-fold higher than that required for cleavage of HGF.

Matriptase Could Function as an Initiator of Matrix-degrading Protease Cascade—Plasmin has long been regarded as the enzyme that converts pro-uPA to active uPA. However, the level of active uPA is not reduced in the urine of mice bearing a targeted disruption of the plasminogen gene (17), suggesting the existence of plasmin-independent pro-uPA activation. Plasma kallikrein (18), trypsin-like proteases from human ovarian tumors (19), a T cell-associated serine protease (20), cathepsins B and L (21, 22), nerve growth factor γ (23), human mast cell tryptase (24), and prostate-specific antigen (25) have also been reported to activate pro-uPA. However, the relevance of these studies of pro-uPA activation *in vitro* is uncertain for understanding roles of these enzymes *in vivo*. During the preparation of this manuscript, activation of pro-uPA was reported by the recombinant serine protease domain of matriptase/MT-SP1 (26). This observation is consistent with our studies using the purified, 70-kDa active matriptase containing both CUB and LDL domains. Fig. 5*A* showed that, after incubation with matriptase, the 55-kDa single-chain pro-uPA was converted into smaller fragments. One of these cleavage products clearly appeared on the protein gel as the 33-kDa molecule, which resembles the size of the active uPA protease (Fig. 5*A*). The cleaved product exhibited enzymatic activity toward the fluorescent peptide substrate, *N-t*-Boc-Leu-Gly-Arg-AMC, for uPA (Fig. 5*B*, compare the *closed circles* with *closed triangles*). This

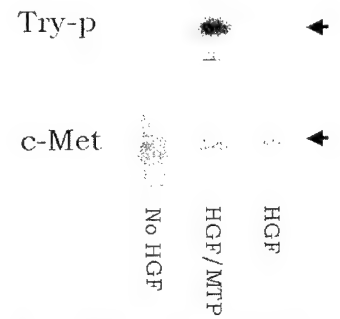

FIG. 3. **Matriptase-cleaved HGF stimulates c-Met tyrosine phosphorylation.** A549 cells were treated in the absence of HGF (*No*), matriptase-cleaved HGF (*HGF/MTP*), or HGF alone (*HGF*) for 5 min at 37 °C. Equal amounts of lysed cell protein were immunoprecipitated with anti-c-Met antibody followed by immunodetection using anti-phosphotyrosin antibody (*Tyr-p*) as described under "Materials and Methods." To control for the amounts of c-Met in each sample, immunoblots were then stripped and detected with anti-c-Met antibody (*c-Met*). The *arrow* points to the 145-kDa β-chain of c-Met under reducing conditions.

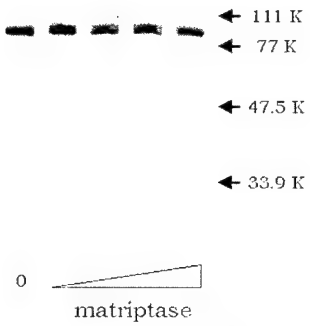

FIG. 4. **Plasminogen is not a substrate for matriptase.** Shown are the silver-stained protein patterns of plasminogen incubated overnight without (*0*) or with increasing amount of matriptase. The highest amount of matriptase used is 8-fold of the lowest amount of matriptase that cleaves HGF.

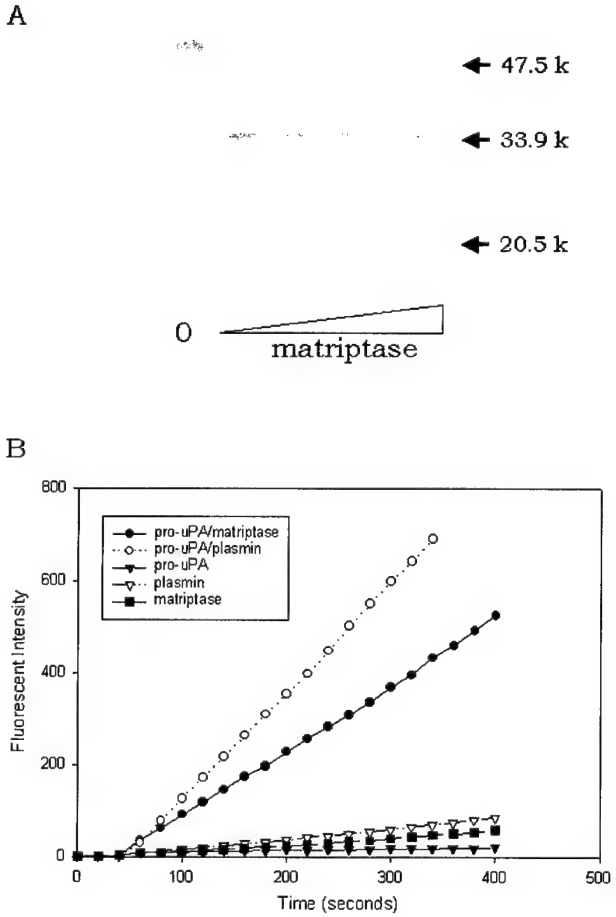

FIG. 5. **Pro-uPA is activated by matriptase cleavage.** *A*, single-chain pro-uPA is converted into two-chain form uPA by matriptase. Pro-uPA was incubated overnight with active matriptase in the absence (*0*) or presence of increasing amount of matriptase. The cleaved products were analyzed by electrophoresis followed by silver-staining. *B*, matriptase cleavage of pro-uPA generates an active protease. Pro-uPA was either incubated for 30 min with matriptase (*closed circles*) or incubated 1 min with plasmin (*opened circles*) prior to the assay. Plasmin (*opened triangles*), uPA (*closed triangles*), and matriptase (*closed squares*) all exhibit low activity.

activity was not derived from matriptase, because matriptase alone only exhibited negligible background activity (Fig. 5*B*, *closed squares*). The same amounts of pro-uPA produced a similar activity after cleavage by plasmin (Fig. 5*B*, *opened circles*). These results suggest that matriptase itself is able to activate pro-uPA and that the CUB domains and LDL receptor domains of matriptase do not interfere with its activation activity.

DISCUSSION

By using the 70-kDa, active matriptase isolated from human milk, we report in this study that matriptase cleaves and converts HGF into a biologically functional factor that can induce c-Met activation and stimulate epithelial cell scattering. In addition, we also noted that matriptase can activate pro-uPA but not plasminogen. These results further support our working hypothesis that matriptase is an upstream regulator of cellular migration and extracellular matrix degradation. Most significantly, these results reveal a novel mechanism in the control of tissue remodeling that involves an upstream epithelial membrane activator and downstream stromal effectors.

Tissue remodeling is a process observed both in physiological and pathologic processes. Two essential changes occur during these processes: 1) an epithelial-mesenchymal transition transforms relatively rigid epithelial cells to the more mobile migratory mesenchymal cells; 2) extracellular matrix degradation opens pathways for the migrating cells. HGF/SF is a potent inducer of epithelial-mesenchymal transition (27); engagement of HGF to its epithelial receptor c-Met triggers various intracellular signaling pathways. HGF is secreted as an inactive precursor by stromal cells, and it is proteolytically activated in the extracellular environment (13). Therefore, activation of HGF/SF needs to occur in the close vicinity of the epithelial cells. uPA and the zinc-dependent metalloproteinases have been proposed to be responsible for the majority of proteolysis of pericellular proteins (28). However, both systems are largely synthesized by the stromal cells and require indirect mechanisms for their recruitment and activation on the surfaces of epithelial cells. Thus, an epithelial-derived protease, like matriptase, could provide a missing link in this process.

Matriptase appears to have selectivity for its macromolecular substrates. In our experiments, matriptase did not cleave plasminogen, despite the high sequence homology between plasminogen and HGF. This selectivity was also reported by others utilizing the serine protease domain of matriptase (26). In the same report, it was also shown that matriptase/MT-SP1 has selectivity for a basic residue at the P3 or P4 site. The sequence at the activation cleavage site of HGF and plasminogen is P4-(Lys)-P3-(Gln)-P2-(Gly)-P1-(Arg) and P4-(Cys)-P3-(Pro)-P2-(Leu)-P1-(Arg), respectively. Thus, the lack of a P3 or P4 basic residue in plasminogen might contribute to its lack of cleavage by matriptase. Alternatively, there might be conformational differences between HGF and plasminogen. The kringle domains of plasminogen contain a Lys-binding site that serves to mediate its localization to fibrin and to cellular surfaces. Plasminogen circulates in the blood in a globular and closed conformation; upon binding to the surface, it shifts to an extended and opened conformation. This conformation change promotes its recognition by its activator and its rapid conversion to plasmin. The kringle domains on HGF/SF also contribute to its binding to its receptor c-Met. However, cleavage activation of HGF/SF does not depend on its binding to c-Met. It is possible that the single chain form HGF/SF exhibits a more open conformation than does plasminogen and that matriptase can distinguish this subtle structural difference.

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Structure-Based Approach for the Discovery of Bis-benzamidines as Novel Inhibitors of Matriptase

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Matriptase, a trypsin-like serine protease, which may be involved in tissue remodeling, cancer invasion, and metastasis. Potent and selective matriptase inhibitors not only would be useful pharmacological tools for further elucidation of the role of matriptase in these processes but also could have therapeutic potential for the treatment and/or prevention of cancers. We report herein the structure-based approach for the discovery of bis-benzamidines as a novel class of potent matriptase inhibitors. The lead compound, hexamidine (**1**), inhibits not only the proteolytic activity of matriptase, (K_i = 924 nM) but also of thrombin K_i = 224 nM). By testing several available analogues, we identified a new analogue (**7**) that has a K_i = 208 nM against matriptase and has only weak inhibitory activity against thrombin (K_i = 2670 nM), thus displaying a 13-fold selectivity toward matriptase. Our results demonstrated that structure-based database screening is effective in the discovery of matriptase inhibitors and that bis-benzamidines represent a class of promising matriptase inhibitors that can be used for further drug design studies. Finally, our study suggested that there is sufficient structural differences between matriptase and its closely related serine proteases, such as thrombin, for the design of potent and selective matriptase inhibitors.

Introduction

Local invasion and metastasis of cancers have been proposed to require imbalanced or unregulated expression of proteases, such as metalloproteases and urokinase-type plasminogen activator (uPA), at invading edges of carcinoma cells.[1−5] In recent years, increasing efforts have been applied to the development of potent and selective inhibitors of these proteases as potential anticancer therapeutic agents.[6−8] Indeed, a number of metalloprotease inhibitors are now in clinical trial for the treatment of cancer.[7]

We have recently characterized a novel, integral membrane serine protease, matriptase (GenBank accession number AF118224), and its cognate inhibitor HAI-1 (hepatocyte growth factor activator inhibitor 1).[9−11] In contrast to most other protease−inhibitor systems, both matriptase and HAI-1 are selectively expressed by cultured breast epithelial cells and cancer cells, but not by fibroblasts or fibrosarcoma cells. In addition to the C-terminal serine protease domain, the N-terminal noncatalytic region of matriptase contains two tandem repeats of a CUB (*C*1r/s, *U*egf, and *B*one morphogenetic protein-1) domain and four tandem repeats of a low-density lipoprotein receptor domain that are likely to be involved in protein−protein interaction.[10] Immunofluorescent staining of cultured breast cancer cells demonstrated that matriptase is concentrated on the cell peripheries at pseudopodia and on membrane ruffles in spreading cells.[9] Of particular interest, matriptase has recently been shown to activate hepatocyte growth factor and single-chain urokinase plasminogen activator.[5,12] Recently, a large-peptide inhibitor of matriptase, ecotin, has been shown to retard the growth of PC-3 prostate cancer tumors in nude mice, further suggesting that matriptase may play a role in cancer progression.[13] Taken together, these data suggest that matriptase may be a central regulator of cell migration and cancer invasion and may provide a novel cancer-associated protease target for design of new anticancer drugs.

We are interested in the discovery and development of potent and selective small-molecule inhibitors of matriptase. A potent, selective, nonpeptide, and drug-like small-molecule matriptase inhibitor not only will be useful as a pharmacological tool to further elucidate the biological functions of matriptase but also may have the therapeutic potential for treatment of cancer through stopping invasion and metastasis. Although large-peptide matriptase inhibitors, such as ecotin, have been reported, drug-like, small-molecule matriptase inhibitors are currently not available. Herein, we report our discovery of bis-benzamidines as a class of matriptase inhibitors through structure-based database search.

Results and Discussion

Homology Modeling. The X-ray structure of human thrombin, entry 1hxe (Figure 1A) from the Protein Data Bank,[14,15] was used as template for building the 3D structure of the protease domain of matriptase using homology modeling. It was shown that when the sequence identity/similarity between the modeled protein and the template is between 30% and 40%, the expected main-chain rms deviation between the modeled and the experimental structures for the protein is about 1.5 Å

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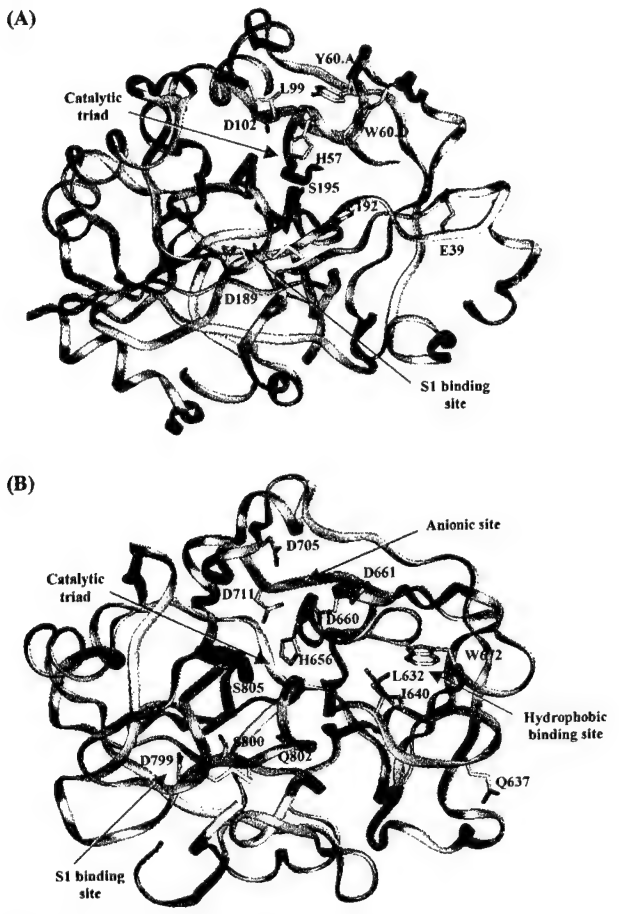


Figure 1. A: Active site residues in thrombin, entry 1hxe in the Protein Data Bank. B: Active site of matriptase, as obtained after homology modeling and refinement using MD simulation in water.

for 80% of residues.[16,17] Since the sequence identity and similarity between thrombin (Figure 1A) and matriptase are 34% and 53%, respectively, and both enzymes belong to the same protease family, it is expected that the 3D structure of matriptase can be modeled accurately. Figure 1B shows the modeled structure of the protease domain of matriptase. By analogy to thrombin, the serine protease domain of matriptase has a catalytic triad positioned on the surface, marked by Ser805, His656, and Asp711 corresponding to Ser195, His57, and Asp102, respectively in thrombin. Consistent with the observation that matriptase prefers substrates with an Arg or Lys as P1 residue,[10,12] a negatively charged residue, Asp799, is located at the bottom of the S1 binding site (Figure 1B). This residue corresponds to Asp189 in thrombin. Ser800 in the S1 binding site in matriptase differs from the corresponding Ala190 in thrombin. Close to the S1 site, Gln802 and Gln637 in matriptase correspond to charged Glu192 and Glu39, respectively, in thrombin (Figure 1). Anionic site residues Asp705, Asp660, and Asp661 in matriptase differ from the corresponding Trp96, Tyr60.A, and Trp60.D, respectively in thrombin. This shows that this site in matriptase is charged while it is neutral in thrombin. A putative hydrophobic S1′ binding site in matriptase is marked by Leu632, Ile640, and Trp672, as shown in Figure 1B, that is similar to the hydrophobic pocket formed by Leu33, Leu41, and Leu64 in thrombin, Figure 1A.

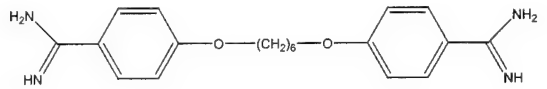
Table 1. Initial Screening of Compound Inhibitors for Matriptase

behavior	number of compounds
over 95% inhibition	15
90−94% inhibition	4
70−89% inhibition	15
40−69% inhibition	13
below 39% inhibition	17
high absorbency	3
increase activity	3

Structure-Based 3D Database Screening. The refined structure of matriptase, obtained from molecular dynamics (MD) simulation, was used for structure-based screening of the NCI database.[18] Since the S1 site is considered to be the primary binding site in serine proteases, it is likely to be a good target site for inhibitor design.[6,19] In addition, two other putative binding sites, the anionic site and the hydrophobic S1′ site, were included in the docking site used for 3D database searching with the program DOCK.[20] Ligands were scored based on the DOCK energy score computed as a sum of the electrostatic, van der Waals, and ligand conformational energy. Since the S1 site of matriptase is negatively charged, the potential inhibitor candidates that target this site should be positively charged in water under physiologic conditions for optimal interactions. Using this hypothesis a total of 69 candidate compounds were selected for testing from the best-scoring 2000 compounds based upon the DOCK program.

Inhibitory Activity Screening. Table 1 shows the results from an initial inhibitory activity screening. Each of the 69 candidate compounds was tested at 75 μM for the inhibitory activity against matriptase: 47 compounds inhibited at least 40% of the protease activity and 15 of them inhibited more than 95% of the protease activity. The 15 compounds that exhibited more than 95% inhibition were analyzed further for their K_i values, as described in the Experimental Section.

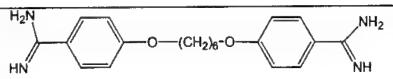
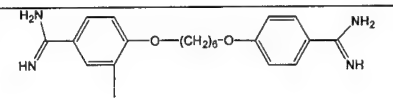
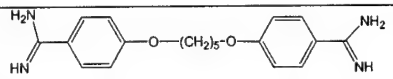
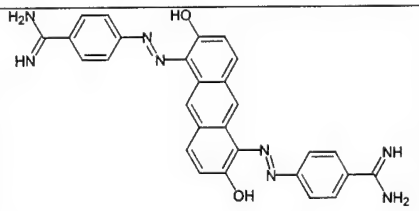
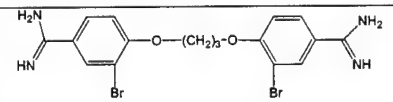
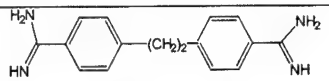
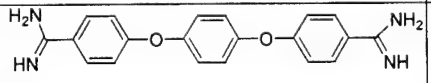
Bis-benzamidines as a Class of Potent Matriptase Inhibitors. One of the compounds that we have identified from our screening is hexamidine, compound **1**, a topical antiseptic. It inhibits matriptase with K_i = 924 nM, which makes it a good lead compound for further optimization. We therefore tested 7 closely related analogues that are available from the NCI database. Their chemical structures and K_i values for inhibition of matriptase enzymatic activity are summarized in Table 2. The K_i values of these compounds ranged from 191 nM to greater than 10 μM. Dixon plots of these inhibitors showed that they behaved as competitive inhibitors to the peptide substrate.



1 (hexamidine)

One important aspect in the design of protease inhibitors is their selectivity. For this reason compounds **1**, **2**, **5**, and **7**, with K_i values below 1 μM, were further evaluated for their selectivity against two other serine proteases, uPA and thrombin. Thrombin is a serine

Table 2. K_i Values Obtained for Bis-benzamidine Analogues of Hexamidine[a]

Cpd	Structure	K_i (nM)		
		Matriptase	uPA	Thrombin
1		924	14,400	224
2		191	1,980	796
3		1,160	N.T.	N.T.
4		4,500	N.T.	N.T.
5		535	1,570	946
6		> 10,000	N.T.	N.T.
7		208	1,950	2,670

[a] N.T. = not tested.

protease that plays a role in blood clotting, and its structure was used as the template to model the structure of matriptase. uPA is another serine protease that was proposed to play a role in cancer invasion and has a high sequence homology to matriptase.[4]

While **1** is 16-fold selective for matriptase over uPA, it is a 4-fold more selective inhibitor for thrombin over matriptase (Table 2). Compounds **1** and **2** have the same structure except for a 3-iodo substituent on one benzamidine phenyl ring. In contrast, **2** is a more selective inhibitor for matriptase; i.e., it displays 5- and 11-fold selectivity for matriptase over thrombin and uPA, respectively. This iodo substituent improved the potency for matriptase by approximately 5-fold, while it decreased the potency for thrombin by approximately 4-fold suggesting that iodo substituents on the benzamidine phenyl ring can improve the potency and selectivity of bis-benzamidines for matriptase. A structural comparison of compounds **1**, **3**, and **6** shows that the length of the linker between the two benzamidine groups plays a role for the inhibitory activity of bis-benzamidines. As the length of the linker decreases, the potency of the inhibitors decreases, which is consistent with our modeled structure of matriptase. Based upon our modeled matriptase structure, the distance between the S1 binding site and the anionic binding site is approximately 21 Å, similar to the length of **1** (20.9 Å) and **3** (19.3 Å) when they adopt a fully extended conformation. Interestingly, although compound **5** has a shorter linker than do **1** and **3**, it is approximately 2-fold more potent than **1** and **3** against matriptase. This suggests that 3,3′-dibromo substituents on the benzamidine phenyl rings improve the inhibitory potency of a compound. Compound **7** is the most rigid compound among **1**, **2**, **5**, and **7** and is also the most selective and one of the most potent inhibitors identified in this study. Despite its shorter linker **7** is as potent inhibitor as compound **2** against matriptase, but **7** has improved selectivity between matriptase and thrombin as compared to **2**. The conformational rigidity of **7** may play a role for its good potency and improved selectivity.

Docking Results. To gain a better understanding of the interactions between bis-benzamidines and matriptase or thrombin, we have docked compounds **1** and **7**, two potent inhibitors with reversed selectivity when tested for thrombin and matriptase. The goal of the docking study is to understand the structural basis of binding and selectivity of these ligands. Docking was done in two steps. First every compound was docked into the corresponding protein using the DOCK[20,21] program. During this docking the ligand was flexible while the protein structure was rigid. To take the flexibility of the protein into account, the complex structure was further optimized using MD simulation

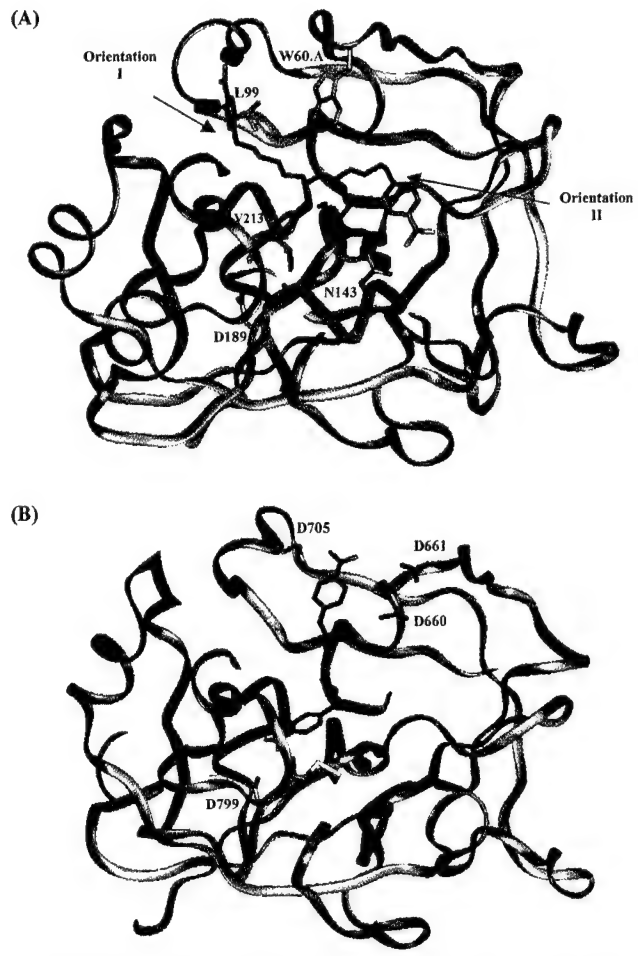


Figure 2. A: Different starting orientations of **1** in the active site of thrombin obtained after flexible ligand docking with the program DOCK. B: Orientation of **1** in the active site of matriptase obtained after flexible ligand docking with DOCK

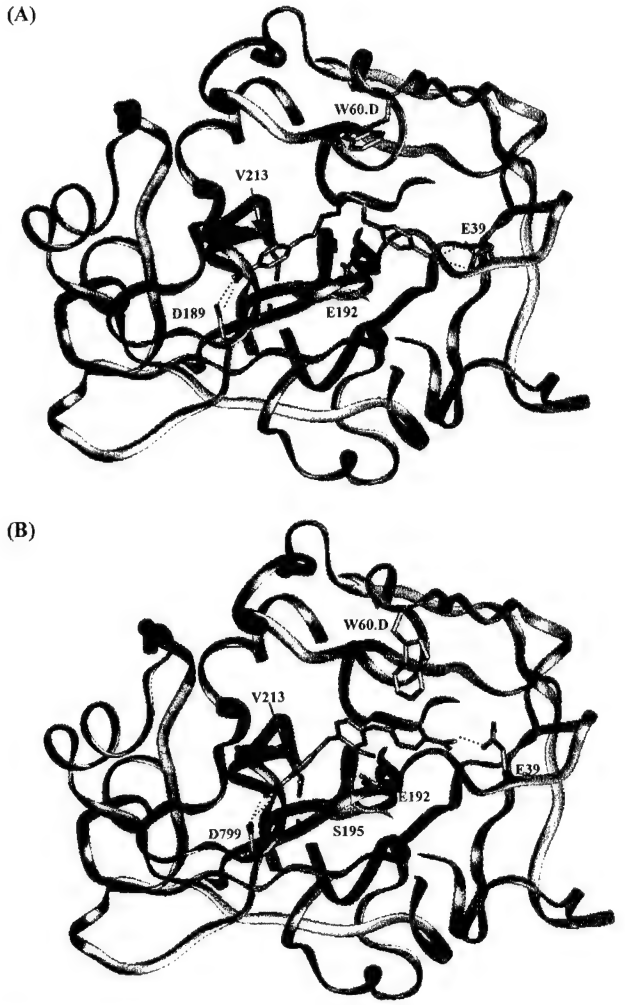


Figure 3. Lowest energy structure of the complex of compound **1** with (A) thrombin and (B) matriptase obtained after refinement using MD simulation with the generalized effective potential.

with a generalized effective potential[22] followed by conventional MD simulation. Docking with DOCK of **1** and **7** showed that these ligands adopt two different binding modes in thrombin (Figure 2A). In both binding modes, one benzamidine ring interacts with the negatively charged S1 binding site. The main difference between these two binding models is that in one binding mode (Figure 2A, orientation I) the second benzamidine fragment interacts with main-chain carbonyl oxygens in a region that corresponds to the anionic site in matriptase, while in the other binding mode (Figure 2A, orientation II), the second benzamidine fragment of the inhibitor interacts with Asn143. Since the automatic docking did not take into account the conformational flexibility of the protein, we investigated whether these two different binding models can converge to the same or similar binding model when the conformational flexibility of the protein is taken into account. For this purpose two parallel simulations were performed starting from the two different binding models of **1**. Using the MD simulation with the generalized effective potential,[22] after 1 ns, both simulations led to the same binding model of **1** in complex with thrombin, Figure 3A. Similarly, simulations of two different binding models for **7** obtained from the DOCK program led to a single converged binding model, Figure 3B. Based on the predicted binding models for **1** and **7** to thrombin, both inhibitors interact with the S1 site through a salt bridge to Asp189, hydrophobic interaction with Val213, and weak hydrogen bond between the amidino group of the inhibitor and the carbonyl oxygen of Ala190 (Figure 3A,B). While **1** interacts with Trp60.D through its linker, **7** interacts with Trp60.D through the second benzamidine fragment. While **1** forms bidentate hydrogen-bonding interactions with the carboxylate of Glu39, **7** forms only monodentate hydrogen-bonding interactions with the carboxylate of Glu39, because the rigid linker in **7** does not allow a favorable orientation of the amidino group to form a bidentate hydrogen-bonding interaction with Glu39. Bidentate hydrogen-bonding interactions between oppositely charged groups are shown to be stronger than monodentate interactions as observed for a series of thrombin inhibitors,[23] which may be one of the reasons why **7** is less potent than **1**.

Docking of **1** and **7** into matriptase using the program DOCK resulted in only one orientation for both inhibitors, Figure 2B. Both binding models were further refined using the same MD protocol as for thrombin. The refined binding models for **1** and **7** are shown in Figure 4A,B. Based upon the predicted models, **1** and **7** interact with the S1 site of matriptase through a salt bridge with Asp799, hydrogen bond with Ser800 OH, and have hydrophobic interaction with Val 824, similar to the interactions with thrombin. However, **1** forms two

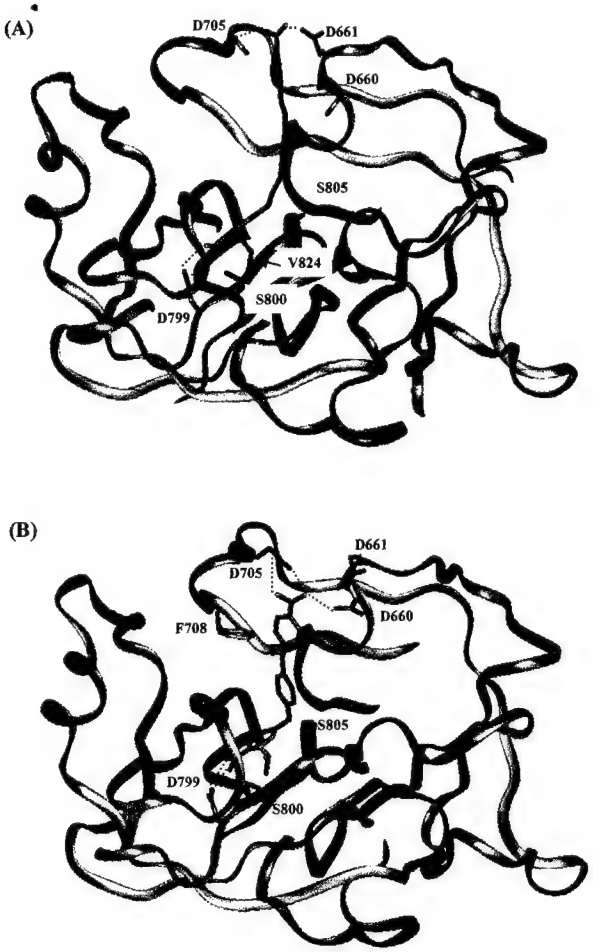


Figure 4. Lowest energy structure of the complex of compound **7** with (A) thrombin and (B) matriptase obtained after refinement using MD simulation with the generalized effective potential.

monodentate hydrogen-bonding interactions with Asp705 and Asp661 in matriptase that correspond to a bidentate hydrogen-bonding interaction with Glu39 in thrombin. However there is no hydrophobic interaction of the linker with matriptase hydrophobic residues, unlike that with thrombin. The lack of hydrophobic interactions of the linker in **1** with matriptase may be one important reason for its lower potency to matriptase as compared to thrombin. Due to its shorter linker, compound **7** was able to form a bidentate hydrogen-bonding interaction with Asp705 and a monodentate hydrogen-bonding interaction with Asp660. These interactions are stronger than that observed in thrombin, which may explain the higher potency of **7** to matriptase than to thrombin. Taken together, our docking studies provide an understanding of the structural basis of a class of novel inhibitors binding to matriptase and to thrombin and can offer a plausible explanation for the selectivity of two potent inhibitors.

Conclusion

We have identified bis-benzamidines as a class of matriptase inhibitors through structure-based database search. The lead compound, hexamidine (**1**) has K_i = 924 nM in inhibiting matriptase. Testing available analogues of the lead compound (**1**) led to the identification of **2** and **7** that are better inhibitors of matriptase with K_i = 191 and 208 nM, respectively. Compound **7** is the most selective compound for matriptase among the compounds tested. It has a selectivity of 9- and 13-fold between matriptase and uPA and between matriptase and thrombin, respectively. Our limited SAR and docking studies showed that the length of linker between the two benzamidine groups, the conformational rigidity of the linker, as well as the substituent(s) on the benzamidine ring(s) play important roles for the activity and selectivity. Differences in the relative position to the S1 site of the anionic site in matriptase versus thrombin can be used to design matriptase-selective inhibitors. The discovery of these small-molecule and nonpeptide matriptase inhibitors provides us with valuable pharmacological tools to further elucidate the biological function of matriptase. Structure-based design and chemical modifications toward improving potency and selectivity of the discovered lead compounds are currently underway and will be reported in due course.

Experimental Section

Homology Modeling and Structure Refinement. The sequence for matriptase was obtained from sequencing data. Templates for homology modeling were obtained by searching the Protein Data Bank,[14] using the program BLAST.[24] The structure of thrombin, entry 1hxe with 34% identities, 53% similarity and 6% gaps, was used as a template for modeling matriptase structure using the program MODELLER.[16] The structure obtained from homology modeling was further refined using the MD program CHARMM.[25] Hydrogen atoms were assigned to the modeled structure using the program HBUILD[26] from CHARMM. The protein was then solvated by inserting it in a 30 Å sphere of water and by deleting solvent molecules with heavy atoms that are at less than 2.5 Å from protein heavy atoms. The MD simulation was done using the all-atom parameter set from QUANTA3.2/CHARMm[27] force field, a constant dielectric $\epsilon = 1$, and constant temperature $T = 300$ K. The leapfrog method with 1-fs time step was applied for numerical integration. Long-range electrostatic forces were treated with the force switch method in a switching range of 8–12 Å. van der Waals forces were calculated with the shift method and a cutoff of 12 Å. The nonbond list was kept to 14 Å and updated heuristically. Solvent waters were kept from evaporating by using a spherical miscellaneous mean field potential as implemented in CHARMM. The solvated protein was energy minimized using 250 cycles of steepest descent and 500 cycles of adopted-basis Newton Raphson methods. This was followed by 100-ps MD simulation.

The structure of thrombin, entry 1hxe from the Protein Data Bank, was prepared for docking in the same way as the structure of matriptase.

Structure-Based Database Search. The refined structure of matriptase obtained from homology modeling, as described in the previous section, was used as the target in a structure-based 3D database search. The program DOCK[28] was used for computer-aided database screening to identify potential inhibitors. Shape and binding energy scoring functions were used to screen and rank the potential ligands. Filters were used to eliminate molecules that have more than 10 flexible bonds, to avoid considering overly flexible molecules, and also discarded molecules with fewer than 10 or more than 50 heavy atoms.

The screening of the large NCI database was done on a Silicon Graphics Indigo2 R10000 workstation. The docking was done in two stages, with the ligand flexibility being considered in both. In the first stage, two minimization cycles, with 50 iterations maximum, were considered for each compound from the database. The best-scoring 10 000 molecules were considered in the second stage, when 100 minimization cycles and 100 maximum iterations per cycle were carried out in order to refine the position of the ligand and its score. The top 2 000 compounds were then considered for selecting potential inhibitors for matriptase by inspection to determine if they contain

ionizable groups that will bind to the S1 site and the anionic site. After these screenings, 69 compounds were selected for further biochemical testing.

Molecular Modeling of the Best-Scoring Ligands. Compounds with K_i values less than 1 μM against matriptase were also docked into thrombin using DOCK with the second protocol as shown above. The orientation of the ligand obtained after docking was used as starting orientation in MD refinement, which was done on a Beowulf cluster of PCs in our laboratory. The active site of the protein−inhibitor complex was solvated by centering the molecule on Ser805/Ser195. A 20 Å radius TIP3P[29] water sphere centered in the origin was then added for solvation. Water molecules closer than 2.5 Å to any protein or inhibitor heavy atom were deleted. The solvated complex was energy minimized using 250 cycles of steepest descent and 500 cycles of adopted-basis Newton Raphson methods. This was followed by 1-ns MD refinement using the generalized effective potential implemented in CHARMM by our group.[22] The temperature of the simulation was 300 K and a 1-fs time step was used for numerical integration of the equation of motion. Long-range electrostatic forces were treated with the force switch method in a switching range of 8−12 Å. van der Waals forces were calculated with the shift method and a cutoff of 12 Å. The nonbond list was kept to 14 Å and updated heuristically. For ligand optimization an annealing protocol was used with the maximum q value[22] for calculating the generalized effective potential of 1.0005; this value was reached from the starting $q = 1$ after 10-ps simulation, followed by 10-ps simulation during which the q value was decreased to 1, which corresponds to MD, and 30-ps MD simulation. This cycle was repeated for the entire length of the simulation. A harmonic restraining force, with a force constant of 0.5 kcal/Å^2, was applied on the protein main-chain atoms that are within 20 Å of the catalytic triad Ser805/195. Residues that were farther than 20 Å from the active site Ser were fixed. This was followed by 1-ns regular MD simulation at 300 K without restraining force on the protein main-chain atoms. During this simulation residues that were farther than 20 Å from the catalytic triad Ser were also fixed. We used the same setup as for the generalized effective potential with the exception that $q = 1$ during the entire length of the simulation.

Materials. Active matriptase was purified from human milk as will be described later. Active urokinase-type plasminogen activator (uPA) was purified by aminobenzamidine-Sepharose 6B (Amersham Pharmacia, Piscataway, NJ) from a partially purified uPA from human urine. Bovin β-trypsin, bovine thrombin, and fluorescent peptide substrates *N-tert*-butoxycarbonyl (*N-t*-Boc)-Gln-Ala-Arg-7-amido-4-methylcoumarin (AMC), *N-t*-Boc-Leu-Gly-Arg-AMC, and *N-t*-Boc-Leu-Arg-Arg-AMC were purchased from Sigma (Sigma Chemical Co., St. Louis). Small-molecule inhibitors were obtained from the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. Other chemicals are all reagent grade.

Purification of Active Form Matriptase. Activated matriptase, in a complex with its endogenous inhibitor HAI-1, was purified from human milk by immunoaffinity chromatography and maintained in its uncomplexed status in glycine buffer pH 2.4, as described previously.[11] Matriptase and HAI-1 were further separated by 10% SDS−PAGE. The proteins were stained by zinc stain kit (Bio-Rad, Hercules, CA). Gels containing the 70-kDa active matriptase were sliced out and eluted using Electro-Eluter (Bio-Rad, Hercules, CA) under nondenaturing conditions (Tris-glycine buffer, pH 8.3). Purified, active matriptase was then stored at −80 °C in acidic solution.

Determination of Inhibitory Activity. Inhibitory activity of compounds against each protease was measured at room temperature using fluorescent substrate peptides in 100 mM Tris-HCl (pH 8.5), containing 100 μg/mL bovine serum albumin. To a cuvette containing 170 μL of buffer were added 10 μL of enzyme solution and 10 μL of inhibitors. After preincubation, 10 μL of substrate was added, and the solution was mixed well by shaking the cuvette. The residual enzyme activity was then determined by following the change of fluorescence released by hydrolysis of the fluorescent substrates in a fluorescent spectrophotometer (HITACHI F4500), with excitation at 360 nm and emission at 480 nm. Peptide *N-t*-Boc-Gln-Ala-Arg-AMC was used as a substrate for matriptase and trypsin, peptide *N-t*-Boc-Leu-Gly-Arg-AMC was used as a substrate for uPA, and peptide *N-t*-Boc-Leu-Arg-Arg-AMC was used as a substrate for thrombin.

Kinetic Screening of Compound Inhibitors. The inhibitory activity of each compound was first investigated by using a fixed (75 μM) concentration both of inhibitor and matriptase. Compounds that exhibited inhibition were then subjected to a further analysis for their K_i values using Dixon plotting. We recorded the rate of hydrolysis in duplicate in the presence of 6−7 different concentrations of each inhibitor. A straight line of the concentration of inhibitor versus the reciprocal values of the rate of hydrolysis was plotted with SigmaPlot software. Two lines were obtained from two unsaturated substrate concentrations; the X value of the intersection of these lines gives the value of $-K_i$.

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Synthesis and Evaluation of the Sunflower Derived Trypsin Inhibitor as a Potent Inhibitor of the Type II Transmembrane Serine Protease, Matriptase

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Abstract: We report here the synthesis of a 14 amino acid long bicyclic peptide, previously isolated from sunflower seeds. This peptide, termed sunflower trypsin inhibitor (SFTI-1), is one of the most potent naturally occurring small-molecule trypsin inhibitors. It is comprised of a backbone cyclized loop bisected with a disulfide bridge. In addition to inhibiting trypsin, the synthetic SFTI-1 is also a very potent inhibitor, with a K_i of 0.92 nM, of the recently derived epithelial serine protease, termed 'matriptase'.

Introduction: Plant derived protease inhibitors serve in the defense mechanisms of plants against pests and plant pathogens (1). These inhibitors can be classified into a number of families based on their active-site structures and their specificities to inhibit the cleavage of specific peptide sequences within proteins. The majority of these inhibitors are classified as serine proteases. One of the well known serine protease inhibitory agents, the Bowman-Birk inhibitor, found in seeds of legumes and other plants, belongs to the Bowman-Birk inhibitor family of small proteins with a MW range of 6000-8000 (2). These proteins inhibit trypsin, chymotrypsin or elastase, depending on the configuration of the reactive site loop within the inhibitor.

Recently, a 14-amino acid peptide, termed sunflower trypsin inhibitor (SFTI-1) was isolated from sunflower seeds (3). SFTI-1 inhibited β-trypsin with an impressive subnanomolar K_i of 0.1 nM, and it inhibited cathepsin G with a comparable K_i (3). SFTI-1 has considerable selectivity; for example, it proved to be 74-fold less inhibitory for chymotrypsin, and was found to be 3 orders of magnitude less inhibitory for elastase and thrombin. In contrast, it had no effect on Factor Xa (3).

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The natural product, SFTI-1 was partially characterized by classical techniques, and its structure confirmed, based on the electron density map of the inhibitor co-crystallized with bovine-β-trypsin (3). SFTI-1 is one of the smallest, naturally occurring plant protein inhibitors reported to date; it has considerably enhanced potency, relative to other peptides of similar length. Its backbone-cyclized peptide structure is additionally stabilized by a cystine disulfide bond. We report here the facile synthesis of SFTI-1. This methodology will also provide for the synthesis of various analogs, with altered inhibitory profiles, relative to various serine proteases of interest.

One such serine protease of interest, is known as matriptase (or MT-SP1), a member of the emerging class of type II transmembrane serine proteases (4,5,6). The mouse homolog of matriptase has also been described and is termed epithin (7). Matriptase /epithin is of considerable interest for the development and pathogenesis of epithelial tissues. Although matriptase is initially synthesized by multiple types of epithelial cells as a transmembrane serine protease, it was isolated originally from human milk in its activated form complexed with its cognate Kunitz type of serine inhibitor (KSPI), the hepatocyte growth factor activator inhibitor (HAI-1). Additionally, matriptase was isolated from human breast cancer cells in culture (4,5,8). Matriptase may function to degrade the extracellular matrix, as well as several cellular regulatory proteins; specifically, it may activate hepatocyte growth factor (HGF) by cleaving its inactive proform, it may activate urokinase by cleaving its zymogen, and it may cleave and activate the protease activated receptor-2, PAR-2 (9,10). Thus, matriptase blockade could potentially modulate cell proliferation, motility, invasion, and differentiation of cells (6,10). We report here the synthesis of SFTI-1 and its potent enzyme inhibitory effectiveness with matriptase, in comparison to its potency for related serine proteases. SFTI-1 thus represents a potentially useful inhibitor for biological studies and therapy of diseases, including cancer.

Synthesis of SFTI-1

Fmoc chemistry based solid phase peptide synthesis methodology was used for the synthesis of the linear peptide RCTKSIPPICFPDG-Rink resin. The total synthesis of bicyclic peptide SFTI-1 is described in Scheme 1. The acid sensitive 4-(2',4'-dimethoxyphenyl-

hydroxyphenyl)-Phenoxy('Rink resin') was purchased from Bachem California Inc. (Torrance, CA, USA). Fmoc derivatives of standard amino acids were obtained from Perkin-Elmer/Applied Biosystems Division. Side-chain protections are as follows: Arg(Pmc), Cys(Trt), Thr(t-Bu), Lys(Boc), Ser(t-Bu), Asp(OtBu). HBTU/HOBt activation of N^{α}-protected amino acids was employed for coupling, and 20% piperidine/DMF was used for Fmoc deprotection. HATU/HOAt/DIEA in DMF was used for backbone cyclization. The crude peptide was purified by reverse-phase high-performance liquid chromatography (RP-HPLC). HPLC conditions: Vydac C4 column (20 X 250 mm); solvent gradient, A, 0.05% TFA in water; B, 0.05% TFA in 90% acetonitrile in water with gradient indicated below; flow rate, 10 mL/min; UV detector, 225 nm. FAB-MS (unit resolution, glycerol matrix) was performed on a VG Analytical 7070E-HF mass spectrometer. Amino acid analysis (6N HCl, 110°C, 24 h) was carried out at the Protein and Carbohydrate Structure Facility (University of Michigan, Ann Arbor, Michigan, USA).

The first amino acid, glycine, was attached to the acid labile Rink resin. Positioning Gly at the C-terminal end minimizes the possibility of racemization on subsequent backbone cyclization. Fmoc-Gly-OH (297 mg, 1.0 mmol) was double coupled with the Rink resin (357 mg, 0.1 mmol) by using 1,3-diisopropylcarbodiimide (1 mmol), 4-(dimethylamino)pyridine (0.1 mmol) and N-methylmorpholine (0.1 mmol) in 3 mL DMF (2 hr, RT). The Fmoc-Gly-Rink resin was dried under a vacuum and treated as a preloaded resin for automated peptide synthesis with an ABI 433A peptide synthesizer using the FastMoc protocol. On completion of the sequence RCTKSIPPICFPDG-Rink resin, the side-chain protected peptide was cleaved from the resin with 20 mL of 1% TFA/DCM (1 min at RT). The solution was collected by filtration and neutralized with NMM in an ice bath. The resin was treated similarly 2 more times with 1% TFA/DCM (5 min at RT) and the combined neutralized solution was evaporated to dryness *in vacuo*. For backbone cyclization, the crude side-chain protected peptide was dissolved in anhydrous DMF (80 mL), and added DIEA (0.6 mmol) and HATU/HOAt (0.30 mmol each) at ice bath temperature. The reaction mixture was stirred at RT for 24 h, neutralized with 30% AcOH and evaporated *in vacuo*. The residue was treated with 95% TFA containing 2.5% each of triethylsilane (TES) and H_2O to deprotect the side chains. Subsequently, for disulfide oxidization, the head-to-tail cyclic peptide was dissolved in 20 mL of water and added dropwise to 250 mL of water solution, which was previously adjusted to pH 8.5 with ammonium acetate

and ammonium hydroxide. The disulfide cyclization occurred spontaneously under the basic conditions while bubbling with oxygen for 6 h, and then stirring overnight at RT. The reaction was quenched by adding AcOH and the solution was lyophilized. The synthetic SFTI-1 was purified by RP-HPLC, Rt = 17.6 min (gradient 20-60% B over 40 min); FAB-MS $(M+H)^+$ 1513.0 (calc. 1513.7). Amino acid analysis: Asp 1.02(1), Ser 0.91(1), Thr 1.15(1), Pro 2.79(3), Lys 0.97(1), Gly 1.15(1), Arg 1.16(1), Ile 1.83(2), Phe 1.02(1).

[Scheme 1]

Enzyme Inhibitory Assays

The 70-kDa activated matriptase was isolated as described previously (5,7,8). Urokinase-type plasminogen activator (uPA) was purified by aminobenzamidine-Sepharose 6B (Amersham Pharmacia, Piscataway, NJ) from a partially purified uPA from human urine. Bovine β-trypsin, bovine thrombin, Bowman-Birk Inhibitor (BBI), and the fluorescent substrates were purchased from Sigma (Sigma Chemical Co., St. Louis). Inhibitory activity of SFTI-1 to proteases was measured at RT in a reaction buffer of 100 mM Tris-HCl (pH 8.5) containing 100 mg/mL of bovine serum albumin, using the fluorescent substrate peptides. To a cuvette containing 170 μL of reaction buffer was added 10 μL of enzyme solution and 10 μL of inhibitor solution. After preincubation, 10 μL of substrate solution was added and the cuvette content mixed thoroughly. The residual enzyme activity was determined by following the change of fluorescence released by hydrolysis of the substrates, using a fluorescent spectrophotometer (HITACHI F4500) with excitation wavelength of 360 nm and emission at 480 nm. Fluorescent peptide N-t-Boc Gln-Ala-Arg-AMC was used as substrate for matriptase and trypsin, peptide N-t-Boc-Leu-Gly-Arg-AMC was used as substrate for uPA, and peptide N-t-Boc-Leu-Arg-Arg-AMC was used as substrate for thrombin. Hydrolysis rates were recorded in presence of 6-7 different concentrations of SFTI-1. The K_i values were determined by Dixon plots from 2 sets of data with different concentrations of substrate.

[Table 1]

Modeling of SFTI-1 in Complex with Matriptase

The homology modeling approach was used to build the 3D structure of matriptase. A search of the protein databank (12), using the program BLAST (13), has identified thrombin (PDB entry: 1hxe) as a good template for homology modeling, with 34% identities, 53% positives, and 6% gaps. The structure of matriptase was built using the program MODELLER (14). The structure derived in this manner was refined by its solvation with TIP3P water molecules, minimization to eliminate bad contacts, and equilibration at 300 K using molecular dynamics simulation with the program CHARMM (15). For docking purposes, only the active site of matriptase was solvated with a 20 Å radius water sphere of TIP3P water, with the center defined by the average of SFTI-1 Cys3 S, Ser191 O, Ser191 C, and Ser191 C. Only the surface residues of matriptase were considered flexible, and the rest were fixed during the simulation. The structure of SFTI-1 was obtained from the protein databank (PDB entry: 1sfi). The starting orientation of the inhibitor is the same as the one found in the X-ray structure with trypsin, 1SFI (3). The docking of matriptase with the inhibitor was performed using molecular dynamics with Tsallis effective potential (16,17), as implemented in CHARMM (15). The simulation was done using the all atom parameter set CHARMM22, the temperature was set to 300 K, the time step for integration was 1 fs, the potential shift parameter was 11000 kcal/mol, and q, the final value of the Tsallis coefficient, was 1.001. The q-annealing schedule was set to 20 ps, for increasing q from 1.0 to 1.001, 20 ps for decreasing q from 1.001 to 1.0, and 10 ps normal molecular dynamics. The total length of simulation was 4 ns. Long-range electrostatic forces were treated with the force switch method in a switching range of 8-12 Å. Van der Waals forces were calculated with the shift method and a cutoff of 12 Å. The nonbond list was kept to 14 Å, and updated heuristically. Solvent waters were kept from evaporating by using a spherical miscellaneous mean field potential as implemented in CHARMM. An NOE restraint was used on the distance between SFTI-1 Lys5 N and Asp185 C of the protein. This restraint kept Lys5 of SFTI-1 bound to the S1 pocket of matriptase; Asp185 is at the bottom of the binding pocket. The maximum value of the distance was set to 4 Å, similar to the distance in the X-ray structure of trypsin complexed with SFTI-1 (3).

Discussion

Endogenous proteases play a pivotal role in the normal cellular physiology of the cell, such as the proteolytic activation of peptide hormones and the activation of message-transmitting peptides and proteins (18). Our efforts are focussed on regulation of a recently identified protease, termed matriptase. Matriptase was initially purified from human milk, but it is also produced by normal and cancerous epithelial cells in culture. Recent studies have suggested that inappropriate expression of its active form has the potential of producing deleterious effect in tissues, contributing to pathogenic states, such as cancer (5,8). This particular serine protease can degrade extracellular matrix proteins, and activate specific proteins, such as HGF, uPA, and PAR-2, by cleaving its inactive pro-form (9,10). These effects are likely to contribute to abnormal cell proliferation, motility, and states of differentiation. In efforts to identify inhibitors of matriptase for experimental purposes, we evaluated the recently identified sunflower derived trypsin inhibitor (SFTI-1), and found it to be a highly effective inhibitor of the enzyme.

SFTI-1 was recently isolated from sunflower seeds, as a complex with trypsin, and its structure was determined by NMR spectroscopy, and by X-ray crystallography (3). Its promising protease inhibitory profile, reported in the original work (3), prompted us to develop the current synthetic methodology for preparation of this 14-amino acid bicyclic peptide, as outlined in Scheme 1. The amino acid backbone was assembled on Rink resin. After resin cleavage, the fully sidechain-protected peptide was backbone cyclized, followed by deprotection of all sidechains. Air oxidation, in weakly basic medium, smoothly provided in good yield the intramolecularly bridged cyclic peptide, SFTI-1. NMR and X-ray studies demonstrated that SFTI-1 has considerable structural rigidity (3), imparted by the intramolecular disulfide bond. The overall conformation and the amino acid sequence was very similar to the reported structures for the equivalent trypsin-inhibitory loop of the reactive site segments within the Bowman-Birk inhibitors (BBI). That segment (-CTKSIPP-) is also very similar to the trypsin inhibitory segment of the mung bean inhibitory protein (19).

[Figure 1.]

Table 1 summarizes the results for a relevant set of serine proteases. The synthesized SFTI-1, just as its natural form, is a potent inhibitor of trypsin with a Ki value of 1.06 nM. In addition, we found that SFTI-1 inhibited matriptase with comparable effectiveness to trypsin. Although the modeled 3D structure of matriptase was built using thrombin as template, SFTI-1 is a poor inhibitor of thrombin (K_i = 5,050 nM). SFTI-1 is also non-inhibitory for uPA, an important serine protease in the extracellular matrix degradative network. This selectivity of SFTI-1, thus, makes it a valuable tool to study the function of matriptase in biological matrices.

On account of the overall similarity of the secondary structure and 3D structure between SFTI-1 and the trypsin inhibitory loop of BBI, we tested the inhibitory activity of BBI to matriptase. A shown in Figure 1, at concentrations of 1.25, 2.5 and 5 μM, BBI significantly reduced the matriptase hydrolytic activity of standard substrates, compared with the SFTI-1 at the same concentration; however, the inhibitory potency of SFTI-1 is 2-3 fold higher than that of BBI.

[Figure 2, Molecular Model]

A molecular modeling study was initiated in order to better understand the structural features that contribute to the high inhibitory activity of SFTI-1. The predicted structure of matriptase complexed with SFTI-1 was obtained after docking using 4 ns molecular dynamics simulations with the Tsallis effective potential, as shown in Figure 2. This structure was compared to the crystal structure of trypsin, in complex with SFTI-1 (3), to investigate the similarities and differences between the interactions of SFTI-1 with these two proteins. In both structures, Lys5 of the SFTI-1 binds to the S1 site of protease; interacting with Asp189 in trypsin or Asp799 in matriptase. Arg2 of the SFTI-1 mainly interacts with the backbone carbonyl group of Asn97 and solvent molecules in the X-ray structure. Arg2 forms an H-bond with the Phe706 main chain carbonyl and interacts with Phe708 and Phe706 sidechains of matriptase through π - cation interactions. Asp14 in SFTI-1 is largely exposed to solvent in both structures; it forms a hydrogen bond with Asn72 in the trypsin X-ray complex structure, but mainly interacts with Arg2 of SFTI-1 in the docked matriptase structure. Phe12 of SFTI-1 is in close proximity with only one hydrophobic residue, Leu99, in the trypsin complex structure, while this residue interacts with Phe708 in the docked complex structure with matriptase. Our docking studies

suggest that SFTI-1 has similar interactions with matriptase and trypsin, and these results provide a rational explanation for the comparative inhibitory potency of SFTI-1 to these two proteases.

The constrained, conformationally rigid structure of the bicyclic peptide, SFTI-1, provides for a promising pharmacophore model towards further development of more specific inhibitors of extracellular matrix serine-proteases, such as matriptase. Synthetic methodologies allow for the design and synthesis of various analogs, based on the homology-modeled matriptase catalytic site. Synthetic approaches are also available now for the design and generation of libraries of back-bone cyclized peptides with bisecting cystine disulfide bridges, as demonstrated for the development of enzyme inhibitory BPTI mimetics (20).

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Table 1. Protease Inhibitory properties of SFTI-1

Protease	K_i (nM)[a]
Matriptase	0.92
Trypsin	1.06[b]
Thrombin	5,050
uPA	500,000

[a]Assay conditions as described in 'Enzyme Inhibitory Assays' section.

[b]Note: Ki of 0.1 nM was reported by Luckett et al (3) using competitive assay conditions.

Figure Legends

Figure 1. SFTI-1 is several fold more potent inhibitor of matriptase than BBI at low μ-molar concentrations. Figure shows the released fluorescence of the peptide substrate resulting from the proteolytic cleavage by matriptase. The assays were carried out as described in the 'Enzyme Inhibitory Assays' section, in the absence of inhibitors (hatched bars), or in the presence of various concentrations of SFTI-1 (black bars) or of BBI (gray bars).

Figure 2. Modeling based structure of matriptase complexed with SFTI-1. For details, see the Experimental Section.

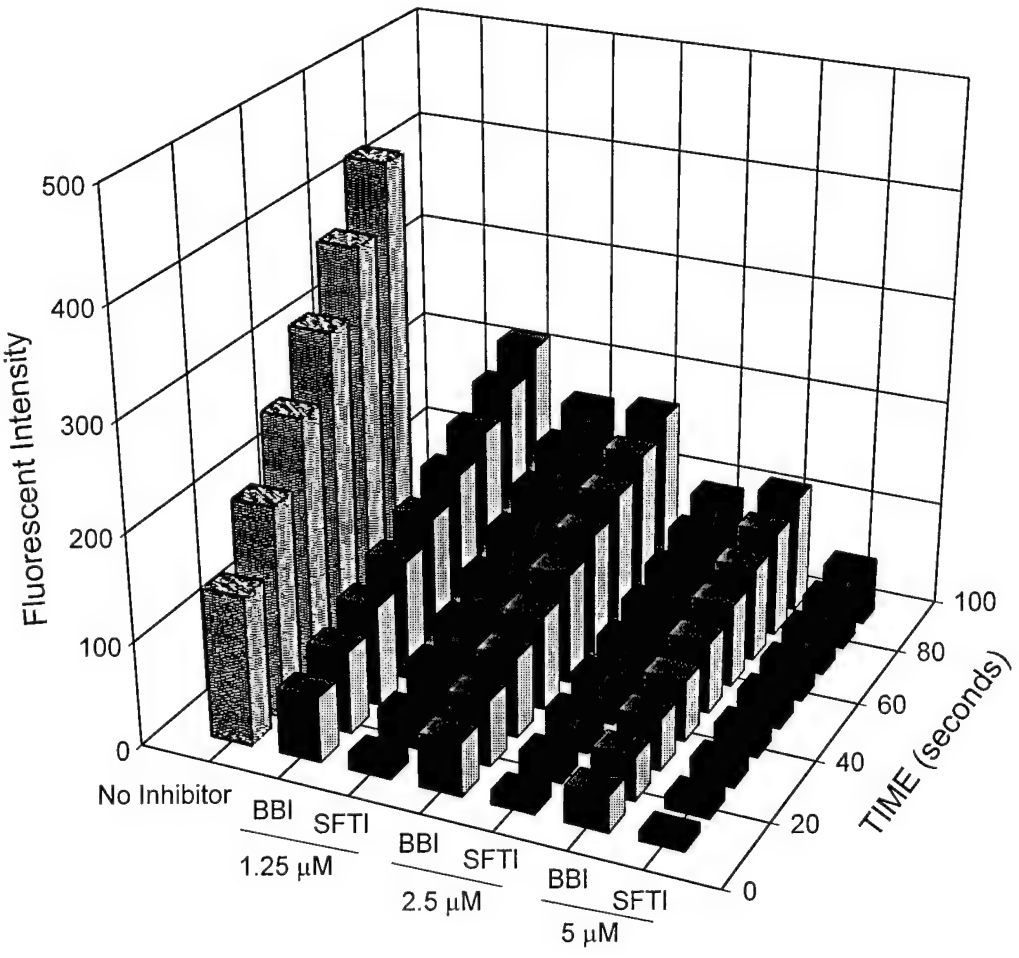

Fluorescent Intensity
500
400
300
200
100
0
No Inhibitor
BBI
SFTI
1.25 μM
BBI
SFTI
2.5 μM
BBI
SFTI
5 μM
0
20
40
60
80
100
TIME (seconds)

Figure 1

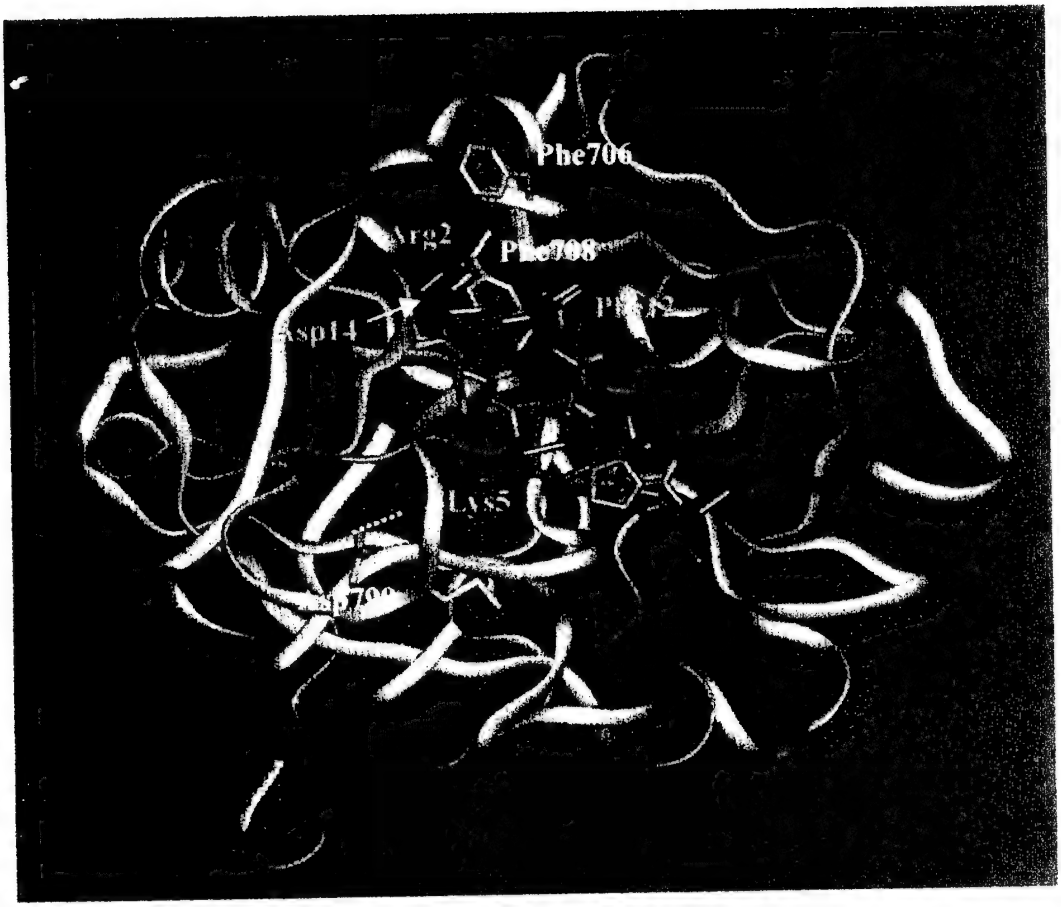
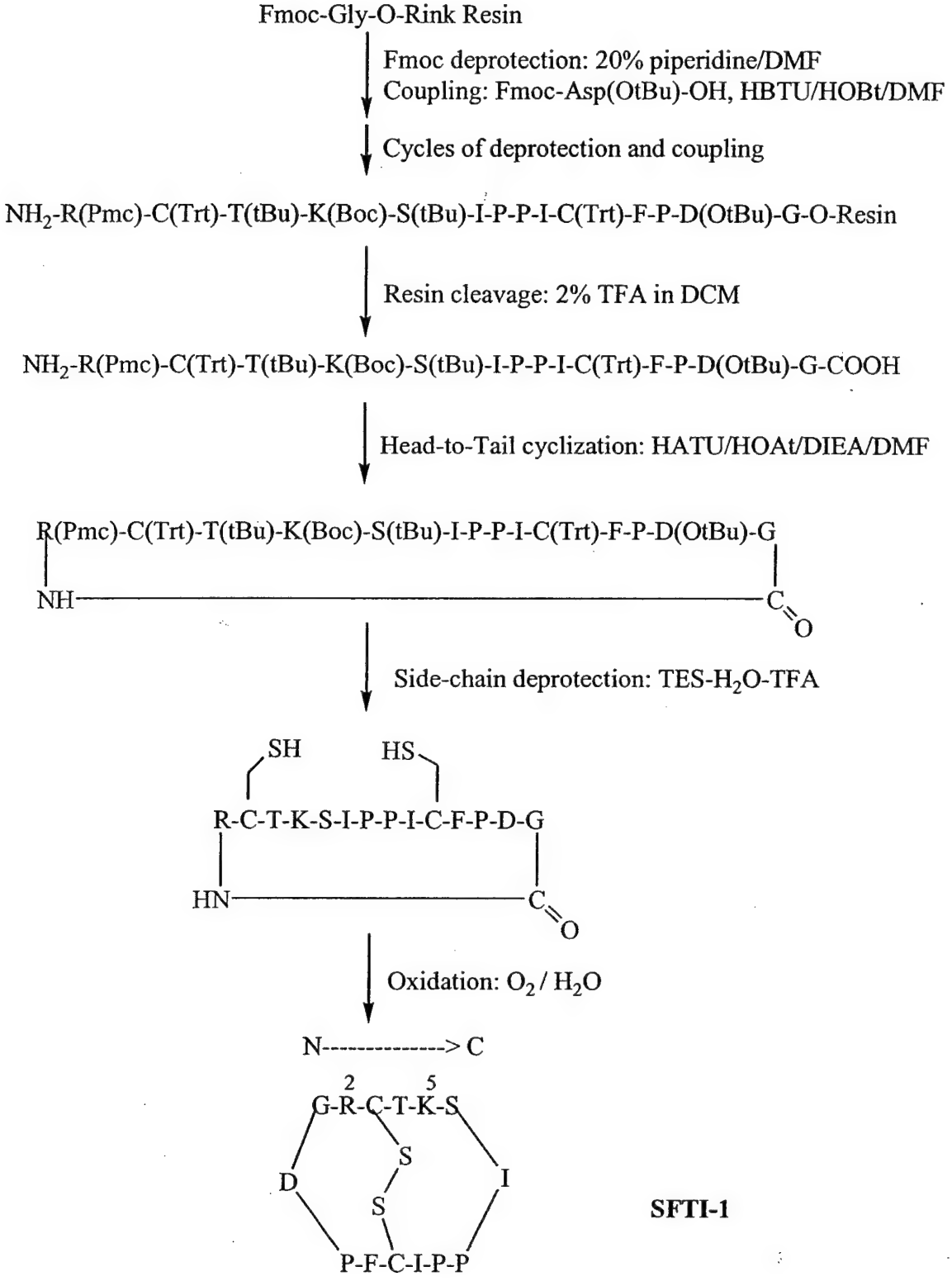


Figure 2



SFTI-1

Scheme 1

Scheme 1. Synthetic route for sunflower trypsin inhibitor **SFTI-1**

Scheme 1 (continued)

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Regulation of the activity of matriptase on epithelial cell surfaces by a blood-derived factor

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Matriptase is an epithelial-derived, integral membrane, trypsin-like serine protease. We have shown previously that matriptase exists both in complexed and noncomplexed forms. We now show that the complexed matriptase is an activated, two-chain form, which is inhibited in an acid-sensitive, reversible manner through binding to its cognate, Kunitz-type inhibitor, HAI-1 (hepatocyte growth factor activator inhibitor-1). Conversely, the majority of the non-complexed matriptase is a single-chain zymogen, which lacks binding affinity to HAI-1, suggesting that matriptase, similar to most other serine proteases, is activated by proteolytic cleavage at a canonical activation motif. We have now generated mAbs specific for the conformational changes associated with the proteolytic activation of matriptase. Using these mAbs, which specifically recognize the two-chain form of matriptase, we demonstrate that matriptase is transiently activated on 184A1N4 human mammary epithelial cell surfaces following their exposure to serum. The ability of serum to activate matriptase is highly conserved across reptilian, avian, and mammalian species. This serum-dependent activation of matriptase on epithelial cell surfaces is followed by ectodomain shedding of both matriptase and its Kunitz-type inhibitor.

Keywords: activation; ectodomain shedding; HAI-1; matriptase; serine protease.

Matriptase is an epithelial-derived, type 2 integral membrane serine protease. It contains two putative regulatory modules: two tandem repeats of a CUB (C1r/s, Uegf and Bone morphogenetic protein-1) domain, and four tandem repeats of a low-density lipoprotein receptor domain [1]. Matriptase was initially characterized by our group as a major gelatinolytic activity in human breast cancer cells [2,3], and subsequently was purified from human breast milk as a complex with a Kunitz-type serine protease inhibitor, termed hepatocyte growth factor activator inhibitor-1 (HAI-1) [1,4]. HAI-1 is a type 1, integral membrane, serine protease inhibitor, containing two Kunitz domains and a low-density lipoprotein receptor domain [5]. Matriptase and HAI-1 are coexpressed, both in human mammary epithelial cells and in breast cancer cell lines. Similarly, the expression of both proteins has been detected in human tissue biopsies; a variety of normal epithelial cells and carcinoma cells were positive [5a]. Matriptase was also cloned independently from human prostate cancer cells by reverse transcription/PCR, and named membrane-type serine protease-1 [6]. Furthermore, the mouse homologue of matriptase, epithin, was cloned from a thymic stromal-derived subtractive cDNA library; epithin is expressed to high levels in thymic epithelial nurse cells [7].

The catalytic domain of matriptase contains an Asp residue at the bottom of substrate binding pocket, suggesting that it is a trypsin-like protease. Indeed, matriptase is able to cleave various synthetic substrates containing Arg or Lys at their P1 sites [1,8]. Three biological substrates have been identified for matriptase, including urokinase-type plasminogen activator (uPA), hepatocyte growth factor (HGF)/scatter factor, and protease activated receptor-2 (PAR-2) [8,9]. Matriptase thus potentially serves as an epithelial-derived, membrane-bound activator for another secreted protease, for a growth factor, and for a cell surface G-protein-coupled receptor. Considering its potent, trypsin-like activity and its potential role in the activation of other important biomolecules, we hypothesized that matriptase activity is likely to be tightly regulated. In this study, we have explored the mechanisms involved in the regulation of the proteolytic activity of matriptase in normal human mammary epithelial cells.

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Abbreviations: CUB, C1r/s, Uegf and Bone morphogenetic protein-1; HAI-1, hepatocyte growth factor activator inhibitor 1; HGF, hepatocyte growth factor; uPA, urokinase-type plasminogen activator; IMEM, Iscove's modified Dulbecco's medium; EGF, epidermal growth factor; ECM, extracellular matrix; PAR-2, protease activated receptor-2.

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MATERIALS AND METHODS

Cell lines and culture conditions

184A1N4 cells (A1N4; from M. R. Stampfer, U.C. Berkeley, CA, USA) [10] and MCF-10A cells (Michigan Cancer Foundation, Detroit, MI, USA) are immortalized, nontumorigenic, human mammary epithelial cells. A1N4 cells were maintained in Iscove's Modified Dulbecco's Medium (IMEM; Gibco BRL), supplemented with 0.5% foetal bovine serum (Gibco BRL), 0.5 $\mu g \cdot mL^{-1}$ hydrocortisone (Sigma), 5 $\mu g \cdot mL^{-1}$ insulin (Biofluids, Rockville, MD) and 10 $ng \cdot mL^{-1}$ epidermal growth factor (EGF; Collaborative Biomedical Research, Waltham, MA, USA).

MCF-10A cells were maintained in 50/50 IMEM/HAM F12 (Gibco BRL) supplemented with 5% horse serum, 0.5 $\mu g \cdot mL^{-1}$ hydrocortisone, 5 $\mu g \cdot mL^{-1}$ insulin and 10 $ng \cdot mL^{-1}$ EGF.

Purification of matriptase from human milk and from the conditioned medium of T-47D breast cancer cells

To purify complexed matriptase (two-chain form), human milk was fractionated by CM-Sepharose chromatography, and the 95-kDa matriptase complex fractions were then loaded onto a rat-derived anti-matriptase mAb 21-9-Sepharose immunoaffinity column, as described previously [4]. Bound proteins were eluted by 0.1 M glycine buffer pH 2.4, and stored in this low pH condition. To purify noncomplexed matriptase (one-chain form) from serum-free T-47D cell-conditioned medium, the complexed matriptase and HAI-1 were first depleted by passing the condition medium through an anti-HAI-1 mAb M58-Sepharose column. The unbound fraction (flow through) was further loaded onto a 21-9-Sepharose column, and bound proteins were eluted by 0.1 M glycine buffer pH 2.4, as described previously [3]. The eluted proteins were stored in low pH to prevent degradation. To investigate the expression of matriptase in human urine, fresh urine was concentrated 50-fold, and then examined by Western blot using mAb M32, a mouse-derived anti-matripase mAb (IgG1).

Diagonal gel electrophoresis

Matriptase samples purified from T-47D cells and human milk were subjected to nonreduced/reduced diagonal gel electrophoresis. In the first dimension, matriptase preparations were boiled in SDS sample buffer in the absence of reducing agents and resolved by SDS gel electrophoresis. A gel strip was sliced out, boiled in SDS sample buffer in the presence of reducing agents, and electrophoresed on a second SDS-containing polyacrylamide gel.

Amino-acid sequence analysis of the 45- and 25-kDa fragments of matriptase

Milk-derived 95-kDa matriptase complexes were purified using a combination of CM-Sepharose chromatography and antimatriptase mAb 21-9-Sepharose immunoaffinity chromatography, as described above. Both 45- and 25-kDa fragments of matriptase were resolved by nonreduced/reduced diagonal gel electrophoresis, as described above, and then transferred to poly(vinylidene difluoride) (PVDF) membranes. The N-terminal sequences of these two fragments were determined as described previously [11] in the Howard Hughes Medical Institute Biopolymer Laboratory & W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

Proteolytic activity assay

The proteolytic activity of matriptase was assayed at 25 °C by incubating matriptase in 200 µL 20 mM Tris buffer pH 8.5, containing 0.1 mM *N-tert*-butoxy-carbonyl-Gln-Ala-Arg7-amino-4-methylcoumarin (Sigma) as a substrate. The rate of cleavage was determined with a fluorescence spectrophotometer (Hitachi, F-4500).

Production of mAbs directed against matriptase

A panel of hybridoma lines, secreting mAbs directed against matriptase, were generated in our previous study [4]. These hybridoma lines were initially selected for mAbs that are able to recognize the 95-kDa matriptase/HAI-1 complex under nonboiled conditions and that additionally recognize the 70-kDa matriptase after boiling.

Immunoblotting

Immunoblotting was conducted as described previously [3]. Proteins were separated by 10% SDS/PAGE, transferred overnight to nitrocellulose sheets (Schleicher & Schuell) or poly(vinylidene difluoride) and subsequently probed with mAb, as indicated. Immunoreactive polypeptides were visualized using peroxidase-labelled anti-(rat Ig) Ig and the ECL detection system (NEN).

Induction of matriptase activation in A1N4 mammary epithelial cells

To serum starve 184A1N4 cells, the cells were plated at 50–60% confluence and maintained for 48–72 h in medium containing 0.5% serum. The activation of matriptase was then induced by incubating the cells with IMEM containing serum. Cells were then scrapped into $NaCl/P_i$, and pelleted by centrifugation (5 min, 1500 *g*). The cell pellets were lysed on ice for 20 min in lysis buffer (1% Triton X-100 in $NaCl/P_i$). Cellular debris were removed by centrifugation (10 min, 14 000 *g*). Equal amounts of protein as determined by the BCA protein micro assay (Pierce), were resolved under nonreducing, nonboiled conditions, by SDS/PAGE and electroblotted onto poly(vinylidene difluoride) membranes (Millipore).

Immunofluorescence

Cells were fixed with 2% paraformaldehyde for 10 min. The two-chain form matriptase was detected with mAb M69 and total matriptase was detected with mAb M32 for 1 h at room temperature. This was followed by a 1 h incubation with 1 : 200 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse (Jackson Immunoresearch). Cells were viewed on a Zeiss microscope, and photographed with Kodak film.

RESULTS

Matriptase is expressed both in single chain and two chain forms

We have shown previously that in human milk, the majority of matriptase is associated with its cognate Kunitz-type inhibitor, HAI-1, forming a 95-kDa complex [4]. In contrast, in T-47D human breast cancer cells, matriptase was mainly present as a 70-kDa, noncomplexed form, although the matriptase/HAI-1 complex was also observed [3]. Because in T-47D breast cancer cells, HAI-1 was detected primarily in its noncomplexed form, this observation suggested that the majority of the matriptase present in the condition media for this cell type lacks binding affinity to HAI-1. Most serine protease inhibitors, with a few

exceptions, first require the cleavage of the target protease at a canonical activation motif, resulting in the formation of the substrate-binding pocket. Only then can these serine proteases associate with the inhibitors that block their activity. Therefore, the lack of interaction between matriptase and HAI-1 observed in T-47D cells, could be explained by the fact that the majority of matriptase is present in the single-chain, zymogen form. In contrast, the complexed matriptase, isolated from human milk, is likely to be in its activated, two-chain form. To test this hypothesis, we isolated complexed matriptase from human milk, and noncomplexed protease from the conditioned medium of T-47D cells. Both matriptase preparations were subjected to nonreduced/reduced diagonal gel electrophoresis (Fig. 1). In this electrophoresis assay, proteins that contain multiple disulfide-linked components will dissociate into their subunits, whereas a single chain will remain as a single entity. As shown in Fig. 1A, the 95-kDa matriptase complex derived from milk was converted to the 70-kDa matriptase and the 40-kDa fragment doublet of HAI-1 under boiled but nonreduced conditions (Fig. 1 panel A, first dimension). When these dissociated proteins were electrophoresed under reduced conditions, the 70-kDa matriptase separated into two groups of polypeptides with apparent sizes of 45-kDa (A chain) and 25-kDa (B chain) (Fig. 1 panel A, 2nd D). In contrast, the noncomplexed matriptase purified from T-47D cells remained as a single chain, with an apparent size of 80-kDa (Fig. 1B). The increase in its size probably results from the reduced rate of migration after treatment with reducing agents. These results suggest that matriptase present in the 95 kDa matriptase/HAI-1 complex consists of two-chains, whereas the noncomplexed matriptase from T47D-conditioned medium is a single-chain protein.

To determine the position of the cleavage site for the generation of the two-chain form of matriptase, the 45-and 25-kDa chains were each subjected to N-terminal amino-acid sequence analyses. The 25-kDa B chain contains the VVGGTDADEGEWP amino-acid sequence at its N-terminus. This sequence begins with the likely cleavage site within the activation motif of matriptase. When the 45-kDa A chain (including two major spots and one minor spot present in the diagonal gel) was sequenced, two overlapping sequences (SFVVTSVVAFPTDSKTVQRT; TVQRTQDNSCSFGLHARGVE) were obtained, and both matched sequences close to the N-terminus of matriptase. These two different N-terminal sequences may be derived from the two major spots of matriptase A chain, and suggest that the different migration rates of the two components result from different N-termini.

HAI-1 binds and inhibits matriptase

It was shown above that HAI-1 can form stable complexes with the two-chain form of matriptase (see also [1,3,4]) (Figs 1 and 2). To demonstrate that HAI-1 inhibits the activity of matriptase, the matriptase/HAI-1 complexes were purified from human milk, as described previously [4], and maintained in pH 2.4 to prevent the association of HAI-1 with matriptase. As the pH of the solution was increased to pH 8.0 and incubated at 37 °C, the formation of 95-kDa matriptase/HAI-1 complex occurred rapidly (Fig. 2A). Binding of HAI-1 to matriptase was reflected by the shift of matriptase from the 70-kDa noncomplexed form, to the 95-kDa matriptase/HAI-1 complex. Noncomplexed matriptase became undetectable by immunoblotting after 30 min of incubation (Fig. 2A). While strong gelatinolytic activity was observed for the 70-kDa two-chain noncomplexed matriptase in a gelatin zymogram (Fig. 2B), only trace amounts of gelatinolytic activity could be detected for the 95-kDa matriptase/HAI-1 complex. This low level of proteolytic activity observed for the 95 kDa

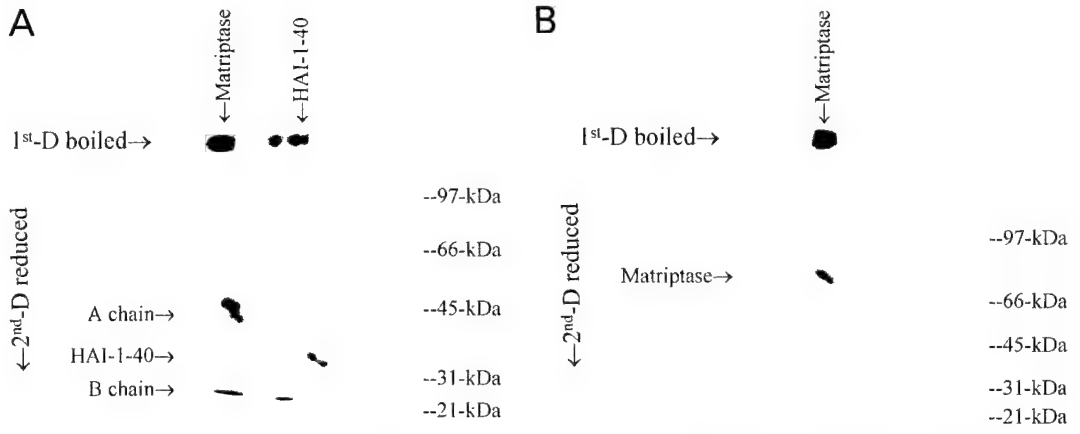
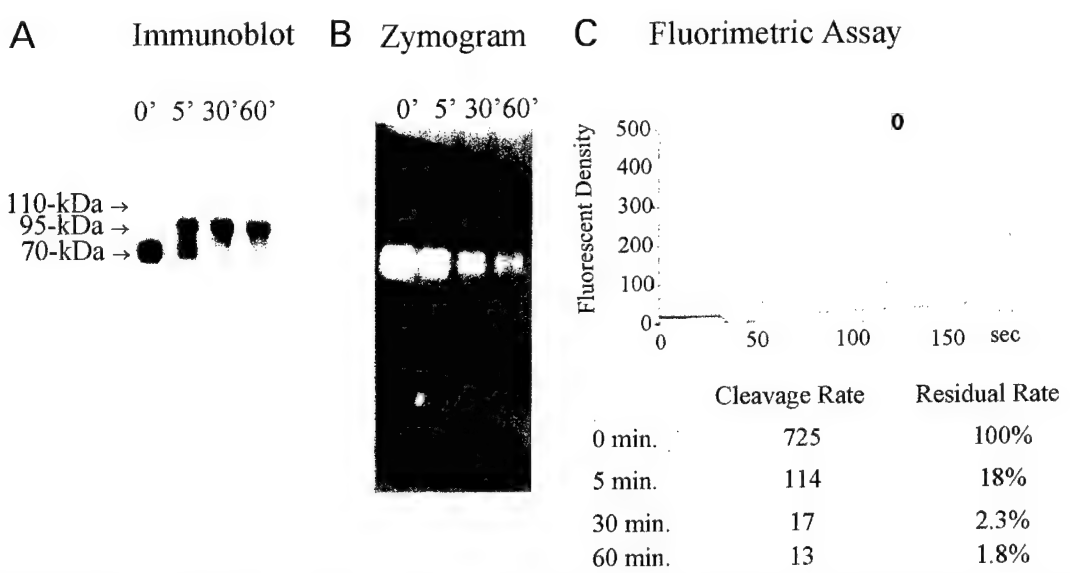


Fig. 1. Nonreduced/reduced diagonal gel electrophoresis of complexed and noncomplexed matriptases. Matriptase was purified by immunoaffinity chromatography using anti-matriptase mAb 21-9 from conditioned medium of T-47D human breast cancer cells and from human milk. These samples were treated with SDS sample buffer in the absence reducing agents, incubated at 95 °C for 5 min, and then resolved by SDS/PAGE (1st-D boiled). Under boiled, nonreduced conditions, the 95-kDa complexed matriptase preparation from human milk was converted to the 70-kDa matriptase and the 40-kDa fragment doublet of HAI-1(A, 1st-D). A noncharacterized copurified protein, observed between matriptase and the HAI-1 fragment, was also seen in this preparation. In the preparation from T-47D cells, only the noncomplexed form of matriptase was purified; no HAI-1 was copurified (panel B, 1st-D). Parallel gel strips were sliced, boiled in 1 × SDS sample buffer in the presence of reducing agents for 5 min, placed on a second SDS gel, and electrophoresed. After these procedures, complexed matriptase (panel A) was dissociated into two components with apparent sizes of 45-kDa (A chain) and 25-kDa (B chain). However, noncomplexed matriptase (panel A) remained as a single chain.



	Cleavage Rate	Residual Rate
0 min.	725	100%
5 min.	114	18%
30 min.	17	2.3%
60 min.	13	1.8%

Fig. 2. Inhibition of matriptase by HAI-1. Matriptase and HAI-1 were isolated from human milk by anti-matriptase mAb 21-9 immunoaffinity chromatography, as described previously [4], and were maintained in an noncomplexed state in elution buffer, 0.1 M glycine pH 2.4. To demonstrate binding to and inhibition of matriptase by HAI-1 this preparation was brought to pH 8.0, incubated at 37 °C for 0, 5, 30, and 60 min, and subjected to immunoblotting using anti-matriptase mAb 21-9 (panel A), gelatin zymography (panel B), and to a cleavage rate assay using the synthetic, fluorescent substrate, BOC-Gln-Ala-Agr-7-amido 4-methylcoumarin (panel C). A milk-derived, matriptase-related 110-kDa protease which does not form a complex with matriptase and HAI-1, is also detected by immunoblotting (panel A) [4].

complex could result from excessive levels of substrate (1 mg·mL^{-1} gelatin) present in the zymogram, as the Kunitz-type serine inhibitors are known to bind to and inhibit serine proteases in a reversible, competitive mode. Furthermore, the rate of cleavage of a synthetic, fluorescent substrate by matriptase was decreased drastically following binding of HAI-1 to matriptase (Fig. 2C). These results provide direct evidence that the two-chain form of matriptase displays proteolytic activity and that binding of HAI-1 results in its catalytic inhibition, which is acid sensitive and reversible.

Production of mAbs which are specifically directed against the active, two-chain matriptase

To investigate the mechanism of activation of matriptase mAbs were generated which can distinguish the activated, two-chain form of matriptase from the single-chain zymogen form of matriptase. Proteolytic cleavage of a single, specific peptide bond in the canonical serine protease activation motif, which transforms catalytically inactive serine proteases into their active forms, results in discrete, highly localized conformational changes [12]. Therefore, mAbs directed against these activation-induced conformational changes of matriptase can differentiate the active form from the latent form of the enzyme. Previously we had generated more than 80 hybridoma lines using the 95-kDa matriptase/HAI-1 complex as an immunogen [4]. In the current study, we further selected two anti-matriptase mAbs, M69 (IgG1) and M123 (IgG1), which are both able to distinguish specifically the two-chain form of matriptase from the single-chain form of the protease. As shown in Fig. 3, mAb M32 detects both purified two-chain and single-chain matriptase forms (lanes 1 and 2). In contrast, mAbs M123 (Fig. 3, lane 3) and M69 (Fig. 3, lane 5) recognize only the two-chain form of the protease; they did not recognize the single-chain form of matriptase (Fig. 3, lanes 4 and 6). In addition to Western blotting analysis, these mAbs are powerful tools to detect specifically the activated form of matriptase in intact cells and tissues.

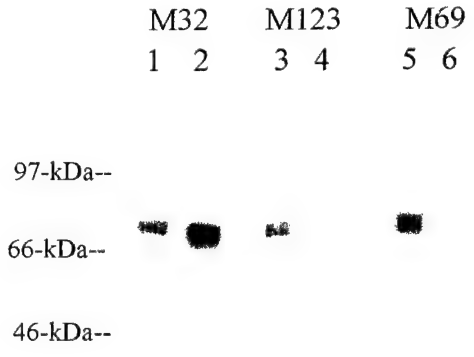


Fig. 3. Production of mAbs directed against the two-chain form of matriptase. Anti-matriptase mAbs, produced in our previous study [4], were subjected to further selection for their differential immunoreactivity against the purified, two-chain form (lanes 1, 3, and 5) or the purified single-chain form (lanes 2, 4, and 6) of matriptase. The majority of these anti-matriptase mAbs, as represented by M32, showed immunoreactivity against both the two-chain and the single-chain forms of matriptase (lanes 1 and 2). In contrast, mAbs M123 and M69 recognized only the two-chain form of matriptase (lanes 3 and 5), but not the single-chain form (lanes 4 and 6). It was noticed that the two-chain form of matriptase had a slightly slower migration rate on SDS/PAGE than the single-chain form.

Transient activation of matriptase by sera in human mammary epithelial cells

The two-chain-specific anti-matriptase mAbs were used together with anti-(total matriptase) mAbs to explore the activation of matriptase. Only negligible levels of the two-chain form of matriptase were detected by Western blotting when nontumorigenic 184A1N4 human mammary epithelial cells were maintained for 2 days under low serum conditions. This observation suggests that most of the matriptase is expressed as the single-chain zymogen form in serum-starved A1N4 cells (Fig. 4A and B, time 0). Exposing the cells to fresh foetal bovine serum results in a sharp increase in the level of the two-chain form of matriptase. This increase in the level of the two-chain form occurred within 10 min of serum stimulation (Fig. 4A and B), and was maintained for up to 7 h, at which time the level of the two-chain form decreased strongly (Fig. 4B). The concentration of serum, rather than the availability of matriptase, was the limiting factor for the activation of matriptase, as the duration of the activation of matriptase depends on the amount of serum added to the cells. Active matriptase was still detectable 16 h after serum stimulation when cells were treated with 5%, instead of 0.5% of foetal bovine serum (Fig. 4C). In addition to the 70-kDa two-chain form of matriptase, also detected in A1N4 cells was the two-chain form of matriptase complexed with HAI-1 (data not shown). Immunofluorescence staining confirmed that exposure to serum induces the formation of two-chain form of matriptase, which is localized at the surface of A1N4 cells (Fig. 5). Serum also induced the activation of matriptase in an independently derived immortalized human mammary epithelial cell line, MCF-10A (data not shown). Interestingly, the T47D breast cancer cells did not increase their level of two-chain form of matriptase in response to serum (data not shown). These results suggest that serum contains a factor which can induce activation of matriptase in human mammary epithelial cells. This factor could be consumed or inactivated by the cells, resulting in the transient activation of matriptase.

Activation of matriptase can be induced by sera from various animals

In addition to foetal bovine serum, sera from human, horse mouse, rat, rabbit, duck, chicken, goat, calf, and even turtle were all able to induce matriptase activation in A1N4 (Fig. 6). These results suggest that a blood-based mechanism for activation of matriptase could be evolutionarily conserved.

Activation of matriptase is accompanied by the release of matriptase and its inhibitor from the surface of cells

Further examination of the expression of total matriptase and its inhibitor was carried out as foetal bovine serum was added to the cells: results showed an inverse correlation between the level of the two-chain form and total matriptase. As the level of the two-chain matriptase was increased in A1N4 cells, the level of total matriptase was reduced (Fig. 4B). Interestingly, as the level of active matriptase diminished, the amount of total matriptase increased (Fig. 4B). Expression of HAI-1 in A1N4 cells after addition of serum, paralleled that of total matriptase (Fig. 4B). As matriptase can be detected in cell conditioned medium, the decrease in the levels of total matriptase, accompanying its activation, could be explained by its release from the surface of cells (ectodomain shedding). Indeed, total matriptase and its two-chain form accumulated in the cell-conditioned medium following stimulation with serum

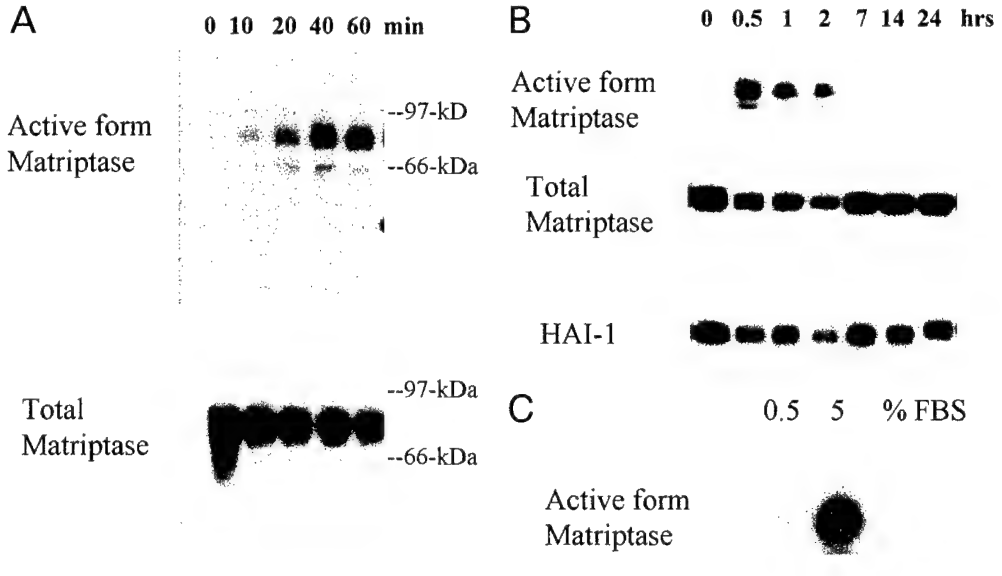


Fig. 4. Transient activation of matriptase by serum. A1N4 cells were maintained for 2 days in low serum. Cells were then stimulated with 0.5% FBS in IMEM (panels A and B) for the times indicated. The cells were harvested, and expression of two-chain matriptase, total matriptase, and HAI-1 were analysed by immunoblotting using mAbs M69, M32, and M19, respectively. In panel C, serum-starved cells were exposed to either 0.5% or 5% foetal bovine serum for 16 h, and expression of two-chain matriptase was determined by Western blotting.

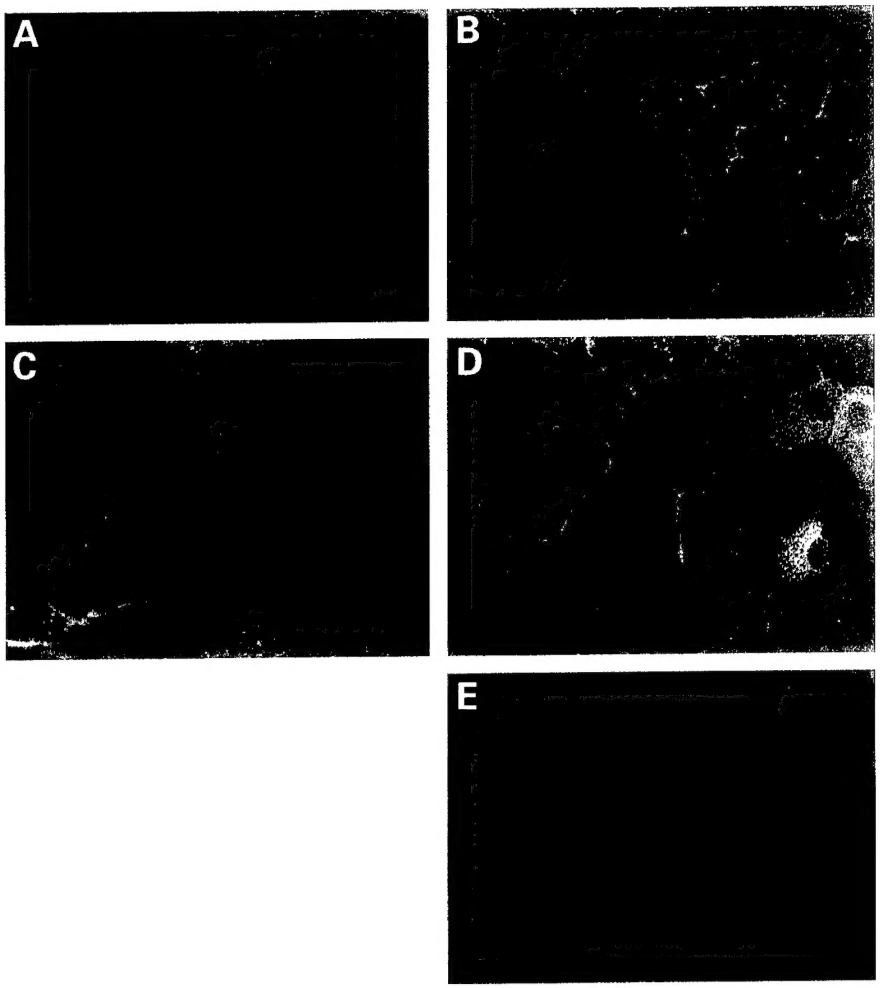


Fig. 5. Immunofluorescence analysis of active matriptase, following serum stimulation of A1N4 cells. A1N4 cells were stimulated with IMEM (A,C) or IMEM containing 1% serum (B,D,E) for 40 min. Non-permebilized cells were incubated with mAb M69 to detect the two-chain form of matriptase (A,B), with mAb M32 to detect total matriptase (C,D), or with fluorescein isothiocyanate-labelled secondary antibody alone (E).

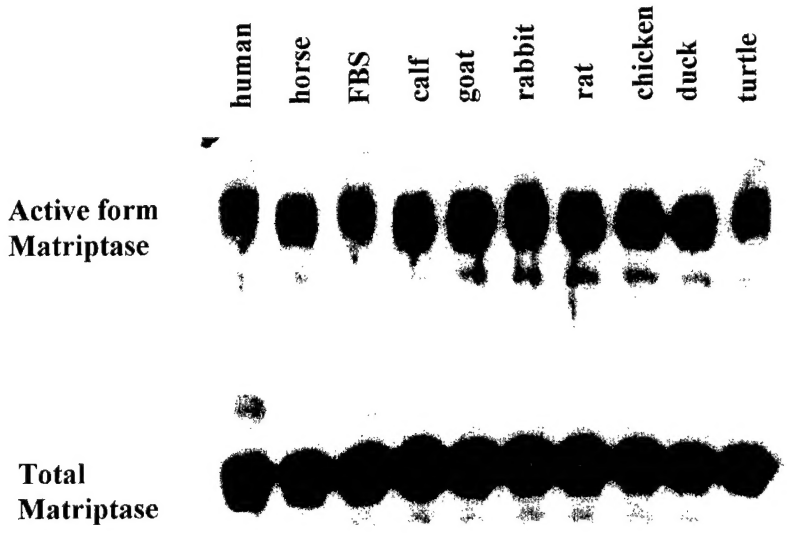


Fig. 6. Matriptase activation is induced by sera from various animal species. A1N4 cells were maintained for 2 days in medium supplemented with 0.5% foetal bovine serum and then stimulated for 1 h with 1% sera from the species indicated. Total cell lysates were analysed by Western blotting for the presence of two-chain form matriptase and total matriptase using M69 and M32 antibodies, respectively.

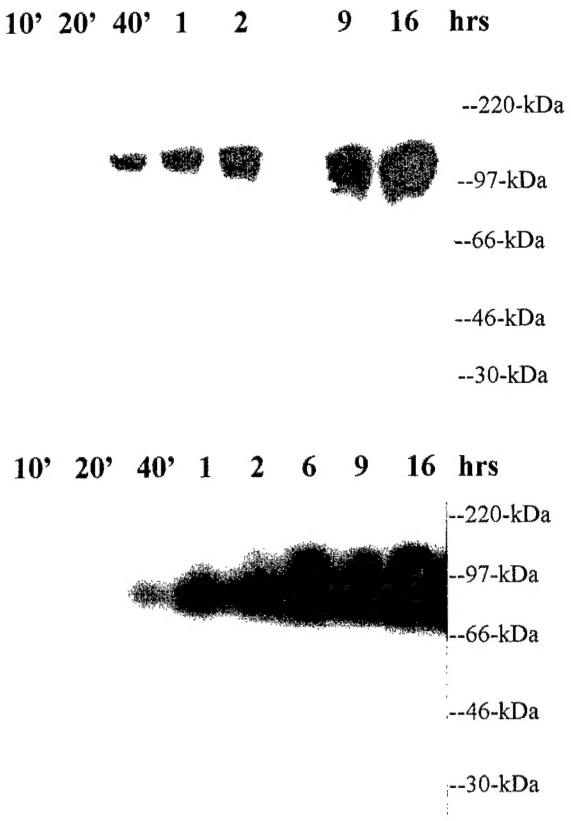


Fig. 7. Activation of matriptase is accompanied by the shedding of matriptase from cells. To investigate the release of matriptase into medium following the activation of matriptase, the conditioned media were collected at the times indicated, following the addition of 0.5% fetal bovine serum to the cells. These media were concentrated and examined by Western blotting for the expression of the two-chain matriptase and total matriptase, using mAb M69 and M32, respectively.

(Fig. 7). These results suggest that in A1N4 cells, serum induces ectodomain shedding, both of matriptase and of HAI-1, in addition to the induction of the activation of matriptase.

DISCUSSION

Matriptase is a potent, trypsin-like protease, which serves as an activator for other proteases, growth factors, and receptors on the surfaces of epithelial cells. Its activity must therefore be tightly regulated. First, we have shown that HAI-1 binds and inhibits the proteolytic activity of the two-chain active form of matriptase. We hypothesized that the proteolytic activation of matriptase would be an irreversible process, similar to most other proteases. Thus its cognate inhibitor, HAI-1, could play a major role in preventing unwanted, prolonged proteolysis once matriptase is activated. The current study describes the discovery of a serum component(s), which can induce the activation of matriptase on the surface of mammary epithelial cells. The serum-induced activation of matriptase is transient, suggesting that this blood-derived inducer can be consumed or inactivated by epithelial cells. Therefore, the availability of this inducer, such as during physiological or pathological states of tissue remodelling, could provide an initial stimulus for the regulation of the activation of matriptase.

Once activated, binding of matriptase to HAI-1 and its shedding from the surface of cells could be essential steps for its inactivation and clearance. Indeed, the 95-kDa matriptase/HAI-1 complex represents the major form of matriptase present in milk [4] and in urine (data not shown), and this complex is likely to be a final product following matriptase activation, shedding, and/or inhibition. Because the shedding of matriptase follows its activation, this ectodomain shedding may serve, along with HAI-1 inactivation, to prevent prolonged retention of proteolytic activity on the surface of cells. Although we used mammary epithelial cells to characterize these regulatory mechanisms, they are also probably involved in the modulation of the activity of matriptase in other epithelial cell types, as the matriptase/HAI system is expressed by various epithelia [5–7].

This blood-based regulatory mechanism for the activation of epithelial proteolytic systems may play an important role in the regulation, maintenance, and repair of the epithelium. Because the epithelium is separated from its underlying connective tissue by the basement membrane, blood vessels do not normally penetrate the epithelium. To reach the epithelium, serum proteins and nutrients must pass through the capillary walls into the surrounding connective tissue and through the basement membrane. Therefore, the activation of matriptase may be regulated by the rate of influx of blood. In some circumstances, such as the lactating mammary gland, proteins from the plasma are transported by transcytosis into the milk. Thus, this large influx of blood through the lactating mammary gland may be expected to promote the activation of matriptase on mammary epithelial cells. Results from our previous studies[4], together with those presented here, indicate that the predominant form of matriptase in human milk is the two-chain form, tightly bound with its cognate Kunitz inhibitor. The discovery of a blood-based mechanism for the activation of matriptase provides a clue for explaining

the increase in the activation of matriptase observed in the lactating mammary gland. Large-scale exposure of epithelial cells to blood components also occurs in kidney, where the expression of matriptase has been reported [6,7]. Thus, serum-dependent activation, HAI-1 inhibition, and shedding of matriptase could also occur in kidney. This hypothesis is supported by detection of 95-kDa matriptase/HAI-1 complex in human urine (data not shown). In addition, matriptase may be important for the process of wound healing, which involves extensive extracellular matrix (ECM) degradation and cell migration. Matriptase and HAI-1 are expressed in epidermal cells, as examined by immunohistochemistry (data not shown). The yet uncharacterized serum component(s) described here could induce the activation of matriptase, which in turn could activate uPA and HGF in the early stages of wound healing. Both uPA and HGF have been implicated in ECM degradation and cell motility, two key events in tissue remodelling and wound healing. Active matriptase then may be quickly inhibited through the binding of HAI-1. As the basement membrane is reformed, the activation of matriptase may then be reduced, as the basement membrane functions as a barrier for blood influx into the epithelium. In contrast with nontransformed mammary epithelial cells (A1N4, MCF-10A), breast cancer cell lines (such as T47D) are insensitive to serum with respect to the activation of matriptase; however, they constitutively maintain low levels of the active form of matriptase, even under serum-depleted conditions (data not shown) [3]. These observations suggest that the blood-derived factor described here might reflect a physiological process. Furthermore, as epithelial cells acquire malignant transformation they may lose this mechanism of transient regulation of the activation of matriptase, but gain constitutive expression of the proteolytic active form of matriptase on their surface (manuscript in preparation).

The mechanism by which serum induces the activation of matriptase still needs to be elucidated. Matriptase contains a canonical serine protease activation motif and a proteolyite cleavage site at Arg–Val, both of which are likely to be required for its activation. When expressed in *Escherichia coli*, the serine protease domain of matriptase can be autoactivated [6]. Although autolytic activation is not the usual case for most serine proteases, it does occur in some instances, such as the complement C1r protease [13,14]. The C1r protease contains a CUB–EGF module, which is thought to be important for the protein–protein interaction and autolytic activation of C1r. Considering that matriptase contains CUB domains, and that autolytic activation of the serine protease domain of matriptase can occur *in vitro*, autolytic activation is thus a potential mechanism for the activation of matriptase. If this is the case, the serum component(s) may act as a C1q-like molecule to transduce the activatory signal, stimulating the autoactivation of matriptase. Alternatively, the serum factor could trigger a proteolytic cascade on the surface of epithelial cells, resulting in the proteolytic activation of matriptase, or it may be a protease that activates matriptase directly.

Activated matriptase is removed from the cell surface by ectodomain shedding, providing an additional means to regulate the amount of protease and the degree of proteolytic activity on the surfaces of epithelial cells. The N-terminal sequences of the two A chains of matriptase were determined to be SFVVTSVVAFPTDSKTVQRT and TVQRTQDNSCSFGLHARGVE; thus, the cleavage sites are located between 266Lys267Ser and 281Lys282Thr (GenBank/EBI Data Bank accession no. AF118224). These results suggest that a still unidentified protease, with cleavage preference between Lys and amino-acid residues containing aliphatic hydroxyl side chains, may be responsible for the shedding of matriptase.

In conclusion, we have described a novel, blood-derived, evolutionarily conserved mechanism for the activation and regulation of an epithelial, membrane-bound, serine protease. The activated matriptase can, in turn, activate pro-uPA, a major stromal ECM-degrading protease system, HGF/scatter factor, a prominent stromal-derived epithelial motility factor in the close vicinity of the cell surfaces, and PAR2, a cell surface receptor. The presence of the Kunitz-type inhibitor, HAI-I, prevents prolonged proteolytic activity of matriptase. Further studies will be required to establish a more direct relationship between matriptase activation, ECM degradation, and epithelial motility. Matriptase may be activated *in vivo* by the contact of blood with epithelial surfaces: downstream effectors of matriptase may play a role in communication between epithelial and stromal cells.

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